

PROTOPLASMA-MONOGRAPHIEN

VOLUME 19

S. C. BROOKS

AND

MATILDA MOLDENHAUER BROOKS

THE PERMEABILITY OF LIVING CELLS

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VOLUME 19

THE PERMEABILITY OF LIVING CELLS

BY

S. C. BROOKS

AND

MATILDA MOLDENHAUER BROOKS

WITH 18 ILLUSTRATIONS

Berlin-Zehlendorf

Verlag von Gebrüder Borntraeger

1941

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S. C. BROOKS

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PREFACE

In writing this monograph we are reminded of the parable of the Joyous Young Man who set out to conquer the world. As Time proceeded swiftly onwards, less and less of the far countries were included in his scope, even fewer and fewer of the outlying districts. He restricted his endeavors more and more, and finally learned that if he would hold his own in his own native district, that was as much as was permitted in his brief life-span to conquer.

In compiling the material in connection with this monograph, it soon became evident that our original plans for making this a comprehensive study of all the factors involved in the study of Permeability, would have to be modified. Indeed, only a fraction of what we desired to present has finally come out of our past hopes. And so we have become convinced that the study of Permeability might be synonymous with the study of Life itself and those factors which determine whether or not a cell survives; that the Unknowns far exceed the Knowns; that the Knowns have led into many fields of Science which are closely related to Biology, Physics, Chemistry, Biochemistry, Anatomy, Physiology, Mathematics and Medicine, but that one can at best pluck only a few of the truths and observations which it has taken thousands of workers to produce.

And so we have finally attempted to crystallize into a critique a few of the factors which we have deemed important in connection with the present-time experimental inquiry into Permeability.

The University of California
Berkeley, California

November, 1939

S. C. BROOKS
M. M. BROOKS

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ADDENDA

Since writing this volume, the exigencies of the War have considerably delayed its publication and meanwhile new methods have added a fund of new facts.

Owing to the impossibility of including a complete bibliography of the past few years only a brief mention of the most important of these experiments from the point of view of ion permeability will be made. These deal with the use of radioactive elements, artificially produced, which have brought to light some surprising conclusions. A first approximation that the speed of penetration of these ions is nearly equal to the rate of free diffusion was discussed by S. C. BROOKS (1937). Since that time confirmation of these results has been found in further experiments not only by S. C. BROOKS (1939a, 1939b, 1940a, b) but by others using different cells.

In order to see if there were any effects upon the metabolism of these cells by the radioactive elements, M. M. BROOKS (1939) used the WARBURG-BAROCROFT respiration apparatus and studied the oxygen consumption of *Nitella*, *Elodea* and various red blood cells. It was found that radioactive NaCl and KCl whose activities ranged from 2.2 mC/liter to 20 mC/liter had no appreciable effect as determined by these methods. MULLINS (1939) investigated the Na-radioactivity of Na*Cl only, by placing it in small glass bulbs immersed in the solution containing *Nitella*. He found that above 1.0 mC/liter there is a decreased rate of penetration of the ions but below this there is no effect. Since it would require extensive treatment to discuss all of these results only a few references will be made here. One important conclusion from the experiments relates to a long-standing controversy as to whether substances penetrate cells as molecules or ions. It had been generally agreed that molecules penetrate cells as molecules, but the question of

ion penetration was left open. The experiments with radioactive ions leave no doubt that ions penetrate by ion exchange, and contrary to the old belief, in minutes or seconds, although in some cases they enter slowly, viz., in several hours. Previous results were dependent upon limitations of the then-existent methods. Since this volume is also in the nature of a critique of the older literature, the tables taken from the experiments produced by the older methods will be left in the volume, hoping that some historical value will be found for them. The reader is referred to the Conference on Applied Nuclear Physics, Cambridge, Mass., October 28 — November 2, 1940, published by the Massachusetts Institute of Technology. This outline contains short abstracts and references to most of the present-day experiments in radioactive elements in relation to permeability. Most of the work deals with the movement of tracer elements into mammalian tissues. Brief mention may be made of the work of HAMILTON and SOLEY on the distribution of iodine in humans, and that of HODGE, MANN and ARIEL, in rabbits; the distribution of potassium, sodium and chlorine in rats and rabbits by FENN (a similar study was made of tissue distribution of sodium by GREENBERG, CAMPBELL and MURAYAMA, 1940); of sodium and potassium in red blood cells by COHN; by WINKLER, EISENMANN and SMITH, and by LARK-HOROWITZ; the distribution of calcium and manganese by GREENBERG; the uptake of phosphorous by tissue cells by BRUES, JACKSON and COHN; the penetration of inorganic arsenic into red blood cells, by HUNTER and KIP; the location of the upward movement of mineral salts in plants by GUSTAFSON and DARKEN, and BENNETT, STOUT and HOAGLAND; and a recapitulation of the ion movement in living protoplasm by S. C. BROOKS.

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EXPLANATION OF SYMBOLS USED IN TEXT

- P = Permeability constant ($\text{GM. cm.}^{-2} \text{sec.}^{-1} (\text{GM. l.}^{-1})^{-1}$).
- P' = Permeability constant where the thickness of the membrane is measured ($\text{GM. cm.}^{-2} \text{cm. sec.}^{-1} (\text{GM. l.}^{-1})^{-1}$).
- V = Volume of a cell
- Π = Osmotic pressure.
- V = Volume of solution occupied by 1 GM (Mol volume).
- R = Gas constant, in liter atmospheres.
- T = Absolute temperature.
- t = Temperature $^{\circ}\text{C}$.
- f = Fugacity.
- P = Hydrostatic pressure.
- p = Vapor pressure.
- b = Non-solvent volume.
- C = Stoichiometric concentration ($\text{GM. (resp. G ions) l.}^{-1}$).
- \bar{V} = Partial molal volume.
- a = Activity.
- A = Activity of substance A.
- (A) = Stoichiometric concentration of substance A.
- GM = Gram moles.
- α = Fractional dissociation.
- μ = Ionic strength.
- z = Valency.
- ϵ = Dielectric constant.
- r = Radius.
- γ = Interfacial tension.
- d = Density.
- Δ = Freezing point depression in $^{\circ}\text{C}$.
- \ln = Natural logarithm.
- \log = Briggs logarithm.
- n = Amount of substance in gram moles.
- A = Area (of membrane).
- x = Distance (along a diffusion path) (y = distance at right angles to x).
- m = Molality.
- cm = Centimeter.
- sec = Second.

l = Liter.

ζ = Zeta potential.

Subscripts

i = Internal (for cells).

e = External.

∞ = Equilibrium.

o = Initial.

t = At time t.

C = Of a cell.

S = Of serum.

CHAPTER I

INTRODUCTION

In order that the various substances which compose a living cell should be properly distributed throughout the cell, it is essential that they pass inwards through the surface of the cell and through the protoplasm. In the same way, metabolic reagents and waste products must pass inwards or outwards through the cell surface and protoplasm.

The rate at which this transport of materials is to occur will depend upon two factors: the driving force, and the resistance which particular parts of the cell offer to the movement of the material in question. It is the latter factor which, strictly speaking, should be referred to as permeability, but both factors must be taken into account if we are to obtain a clear picture of the mechanism by which import and export materials are determined in living cells.

Permeability may be defined as the rate of movement of a substance through the permeable layer under a given driving force. This definition involves two concepts which must be kept sharply distinguished: on the one hand, permeability, — a property of a membrane or region of the cell: on the other hand, the driving force which may be quite independent of any property of the membrane. To these two concepts correspond two others, which must similarly be clearly separated in our treatment. Driving force measures how far short of equilibrium the system is: at equilibrium for any given molecular species the driving force for that species must be zero. Permeability measures the rate at which equilibrium is approached, that is, what fraction of the total change necessary for the attainment of equilibrium is accomplished in unit time.

Plasma and other semi-permeable membranes

The question immediately arises as to where in the cell the semipermeable layers are located. Is there, for instance, a plasma membrane, forming the outermost layer of living cells, and responsible for the typical permeability relations of the cell? Are nuclei and vacuoles surrounded by similar membranes? Or, on the other hand, does the cytoplasm or nucleoplasm have equal permeability throughout its whole volume? Without presenting detailed evidence in this place (See e.g., Chapter I, by CHAMBERS, in Harrow & Sherwin) we may say that there is good reason to conclude that at least some of the interfaces of living cells (e. g., superficial and vacuolar) are considerably less permeable than the bulk of the protoplasm, and usually dominate in determining the normal activities of cells and the outcome of experiments. The influence of the permeability of various parts of the cell other than interfaces, may usually, though possibly not always, be neglected. We shall, therefore, assume, in the absence of evidence to the contrary, that the interfaces at the outer boundary of the cytoplasm and between at least some of the various portions of the living cell, are the seats of characteristic permeability and may be thought of as semi-permeable membranes. Only such living membranes, for which we shall use the general term "protoplasmic membranes", about whose permeability we have any considerable knowledge are the superficial or plasma membrane and the vacuolar and nuclear membranes. A complete picture of the permeability relations of organisms or even of many cells must include excreted and apparently non-living membranes, such as plant cell walls, animal cuticle and the like.

Driving forces:

Activity gradients and electrical forces. The underlying cause of the movement of solutes and water in living organisms is undoubtedly concentration gradients, or, more properly, activity gradients. Secondly, the activity gradient of one substance may give rise to an electrical potential gradient, which in turn may act as a driving force upon some other substance. Therefore, in considering the forces acting to drive a given substance through a membrane we must include not only its own activity gradient but also the possible effects of potential gradients across

the membrane. These two forces (besides mechanical movement of the solution as a whole) seem to be the only ones possible.

Activity gradients. Substances in solution do not necessarily behave as would be predicted on the basis of their stoichiometric concentration, but display properties proportional to the activity (LEWIS, 1907)¹. Activity has no dimensions and is proportional to a fugacity ratio as shown by the equation $a = f/f^0$ in which f^0 is the fugacity of the substance in a chosen standard state. Since f^0 by definition is equal to one, the activity, a , is numerically equal to the fugacity, f , of this equation. The fugacity measures the tendency of a substance to escape from the phase in which it is.

In the case of a substance in solution, the fugacity is the tendency of that substance to leave the solution. Fugacity has the dimensions of a force, and it may be measured by such properties as vapor pressure, correction being made for the fact that the vapor is an imperfect gas (LEWIS and RANDALL, 1923). Since there is a direct relation between fugacity and molal free energy, any measure of the latter will serve as a measure of differences in fugacity. Measurement of electrode potentials is an example. Since the equation connecting partial molal free energy, \bar{F} , and fugacity, f , has the form

$$\bar{F} = RT \ln f + B,$$

where B is a function of temperature only, it is possible to obtain from the known values of \bar{F} the *ratios* of f in two different systems, A and B , since $F_B - F_A = RT \ln \frac{f_B}{f_A}$.

The activity of any substance in a selected standard state is, by definition, equal to unity. In the case of water it is more convenient to choose pure water at the given temperature and external pressure as the reference state, and to assign arbitrarily to water in this state an activity of one. For solutes other standard states have been used. For non-electrolytes the standard state is that state at infinite dilution, while for strong electrolytes the state is that concentration at which, for example, a univalent salt yields equality of the product of the ion activities and that of the undissociated molecules. This is expressed by the equation.

¹) See also LEWIS and RANDALL (1923), especially Chapters 17 and 22.

$a_+ \cdot a_- = a_2$, where a_+ , a_- and a_2 refer to the cation, the anion and the undissociated molecule respectively. The quotient $\frac{a}{e}$ is the activity coefficient, γ , of the ion in question.

Either fugacity or activity may be used to express the force which produces diffusion. Diffusion underlies the movement of water and solutes. Concepts of activities, fugacities and activity or fugacity gradients are of general applicability, and as such, may be used to unify, under one method of treatment, the first and most fundamental type of driving force which results in the passage of materials through protoplasm. Thus, when water passes through a membrane from a dilute to a concentrated solution, it is in reality moving from a region of higher activity to one of lower activity. A solute diffusing through a membrane from a concentrated to a dilute solution is also moving from a region of higher activity to one of lower activity. In the first case, the movement of water is generally known as osmosis, and although not all physical chemists take this view, it seems best to us to apply the term osmosis to the essentially similar movement of a solute when it diffuses through a membrane. The distinction between solvent and solute is at best artificial.

Whenever water or dissolved substances pass through a membrane along their activity gradients we shall, therefore, speak of *osmosis of water or dissolved substances*, and of *endosmosis* or *exosmosis* according as the water or solute is passing into or out of the cell.

In order to express the permeability quantitatively, it seems desirable to reduce the activities to a common basis for solutes, molecules and ions. We can do this by using a suitable term to express activities in terms of molality as corrected by the activity. This is discussed further elsewhere.

There is another effect of simultaneous movement of solute and solvent on each other's rate of movement. The effect discussed in the previous paragraphs is one which affects the driving forces, and is proportionate at any moment to the activity gradients existing at that moment. In addition it is also probable that the rate of movement of one substance affects the simultaneous rate of movement of all other substances in a given solution. To take an extreme case, let us imagine a narrow cylinder containing a sugar solution whose concentration increases regularly from a point A to

a point B. If the solution as a whole is stationary, a certain number of sugar molecules will pass a point M between A and B during every second going in the direction from B to A. If now we introduce at A a steady slow stream of sugar solution having a concentration equal to that at A, and allow the solution to flow off at B, it is obvious that fewer sugar molecules will pass the point M going towards A, or even that the direction of movement of sugar may be in the opposite direction. A mass flow from B to A, similarly produced would greatly increase the number of sugar molecules passing M each second. Since mass flow is simply a statistical result of the individual velocities of the molecules, the same qualitative effects should occur regardless of the speed of flow or the diameter of the cylinder so long as solute and water form a common phase: in the case of living cells the exosmosis of water might be supposed to retard the simultaneous endosmosis of solute, and *vice versa*. The magnitude of this effect in living cells is hard to predict, since it is conceivable that water and solute may be in separate phases, or that both may be dissolved in some common solvent which constitutes a part of the protoplasm and is stationary. In any event the ex- or endosmosis of solute probably has very little effect on the simultaneous movement of water into and out of living cells, because the mole fraction of solute present is ordinarily very low. Until our quantitative data are very much more exact, the effect of the osmosis of one substance on the rate of osmosis of others must be considered to be negligible in all or in most cases. When there is rapid osmosis of water, it is conceivable that its effects on the simultaneous osmosis of solutes will be perceptible. (See TAMMAN, 1892; VAN'T HOFF, 1892).

Electrical forces : Potential gradients. If the substance in question consists of ions, or if the independently moving units (such as crystals, micelles, molecules, or columns of fluid in a capillary space) carry an electrical charge, then their direction and rate of motion will be affected also by potential gradients. The charge referred to may be due to ionization or to the formation of an electrical double layer, which gives rise to the so-called "zeta" or "electrokinetic" potential. In either case the movement of the charged substance must tend to annul the potential gradient which causes that movement (LE CHATELIER's Theorem). A full discussion of electrokinetic phenomena lies outside the province of

this book (See for example, KELLER, 1932, and ABRAMSON, 1934). It seems probable, however, that permeability of living cells does not involve cataphoresis (the electrophoretic migration of particles in a fluid) to any great extent. Possible exception may be made of the smaller non-electrokinetic molecules, which, according to KELLER, are considered to have an electrokinetic potential. Electroosmosis of water and iontophoresis of ions almost surely occurs. These will be more fully considered in connection with the respective classes of substances.

The relative importance of activity and potential gradients as driving forces cannot at this time be determined with finality. KELLER (1932) believes that the latter are extremely important for all classes of substances, while LUCKÉ and McCUTCHEON (1932) in reviewing permeability to water consider only activity gradients (osmotic pressure gradients) to be significant. We incline to the view that electrical forces are negligible in the case of non-electrolytes, significant, though of secondary importance for water, but of predominant importance for ions, especially mineral nutrients.

Equilibrium

A cell in equilibrium internally and with its environment and with respect to all substances would be a dead cell. All living cells are and must be in a non-equilibrium state with respect to at least some substances. Where this state is maintained essentially constant by a steady flux of reactions we may speak of it as being in dynamic equilibrium, or better, in a steady state. It is particularly important that we deal with the *rates* at which transport of material across cellular or intracellular surfaces takes place, rather than to confine our attention to presumed equilibria, because such presumed equilibria are so often merely steady states. Presumed equilibrium is usually spoken of in the literature as "equilibrium", i. e., when there is no more change in ratio of concentration of a solution inside as compared with that outside the cell. This ratio may be equal to 1, or greater or less than 1. Only when it is equal to 1 is a true equilibrium attained. In the case of the other conditions, a "steady state" or presumed equilibrium exists. Cellular activities are usually rapid enough to prevent the establishment of equilibrium for many substances; growth and metabolism are constantly acting

against attainment of equilibrium, and for many substances to which the cell is relatively impermeable, activity gradients will always exist, and equilibrium be far from attainment. Classical thermodynamics, insofar as it is based on the study of equilibria, may, in such cases, be of little use in unravelling the complicated group of interacting factors which determine the material exchange between a living, growing cell and its environment.

In all cases clear distinction must be made between steady states or "dynamic equilibria" which are frequently encountered and true equilibria, which are relatively uncommon.

A living cell may be defined on the basis of its thermodynamic properties as a system not in equilibrium, which converts the forms of energy of its medium into forms of energy acting against the attainment of equilibrium. (See BAUER, 1922).

The quantitative definition of permeability

It has already been pointed out, and should be emphasized, that permeability measures the rate of approach to equilibrium. As such we should aim always to express permeability as a definite constant. This means of expressing the rate at which a process approaches equilibrium will be familiar to many in the form of the unimolecular reaction isotherm. Suppose a substance is initially present in concentration C_0 ; suppose further that a definite fraction of the molecules present at any moment disappears (by some type of reaction) in each unit of time. The rate of disappearance is then

$$-\frac{dc}{dt} = k \cdot C_t \quad \text{I}$$

where C_t is the concentration at time t . The rate constant, k , may be evaluated from either of the two equations derivable from this, viz.:

$$k = \frac{1}{t} \ln \frac{C_0 - C_t}{C_0} \quad \text{II}$$

or

$$C_t = C_0 \cdot e^{-kt} \quad \text{III}$$

The latter equation shows clearly the exponential nature of the relationship.

If the substance in question is being transformed molecule for molecule into some other substance whose ultimate and mo-

mentary concentrations we may call C_∞ and C_t respectively, the corresponding equations will be

$$\frac{dc}{dt} = k(C_\infty - C_t), \quad \text{IV}$$

$$k = \frac{1}{t} \cdot \ln \frac{C_\infty}{C_\infty - C_t} \quad \text{V}$$

and

$$C_t = C_\infty (1 - e^{-kt}) \quad \text{VI}$$

which are also forms familiar to most readers.

Turning now to permeability we find it measurable, like a unimolecular reaction, in terms of an approach to equilibrium. But unlike a reaction, which deals only with concentrations, permeability involves capacity factors such as the areas of membrane and the volume enclosed by it. The first of these factors, i. e., area of membrane or diffusion path is cared for by equations such as those derived by FICK for diffusion. FICK's original equation (1855) for the diffusion of a solute was based upon FOURIER's for heat conduction, and in its differential form is

$$\frac{dS}{dt} = -Q \cdot k \cdot \frac{dy}{dx} \quad \text{VII}$$

where S is the amount and y the concentration of dissolved substance, Q is the cross sectional area of the diffusion path, x is distance in the direction of diffusion, t , time, and k the diffusion constant. Therefore

$$k = -\frac{dS}{dt} \cdot Q^{-1} \cdot \left(\frac{dy}{dx}\right)^{-1} \quad \text{VIII}$$

The right-hand half of this equation is seen to represent the amount of substance diffusing per unit of time through unit area under unit concentration gradient.¹⁾ The analogy between the differential form of this equation and that of the unimolecular reaction isotherm is evident. The only essential difference lies in the introduction of Q , the cross-sectional area of the diffusion path in FICK's equation. FICK considered this equation applicable to the diffusion of a solute through pores in a membrane, provided that the pores had uniform diameters throughout their lengths, and that there was no specific attraction or repulsion of components

¹⁾ FICK's notation is here followed.

of the solution by the membrane material. The effects of these factors were formulated by FICK, but need not be discussed here.

If diffusion to and from the membrane is relatively so fast as to be negligible we may set up for diffusion of solvent or solute through the membrane a similar equation (using the notation of this book):

$$P' = -\frac{dn}{dt} \cdot A \cdot \frac{da}{dx} \quad \text{IX}$$

Here P' is the diffusion constant within the membrane, and the driving force is assumed to be an activity gradient only. Since we do not know the thickness of any plasma membrane with any accuracy, and since furthermore we do not even know that such membranes are homogeneous throughout their whole thickness, it seems best for the present to deal only with the total differences in activity across the membranes. For practical purpose therefore we shall ordinarily use the permeability constant P defined by the equation:

$$P = \frac{dn}{dt} \cdot A \cdot \Delta a \quad \text{X}$$

The quantities in these equations must be expressed in some suitable units in order to give a definite numerical value for P . It is desirable that these should be equally applicable to all classes of substances, and we, therefore, choose in preference to formulas using other types of units (e. g., cubic micra, atmospheres), one employing only gram moles (or gram ions) and c. g. s. units. There has been no general agreement as to which c. g. s. units to use, and we, therefore, arbitrarily define permeability in the sense of the first equation, *as the amount of substance in gram moles (or gram ions) which penetrates through one square centimeter of membrane surface in one second under an activity gradient of 1 Gm (or gram ion) per liter per centimeter thickness of membrane* or $P' = \text{GM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \cdot (\text{GM} \cdot \text{l}^{-1} \text{cm}^{-1})^{-1}$ ¹⁾ where P' is the permeability constant. Since 1 liter = 1000 cm³, P' has the dimension cm² · sec⁻¹ or l² · t⁻¹. The thickness and structure of living plasma and vacuolar membranes being unknown,

¹⁾ Strictly speaking this relation is true only at 4° C. and atmosphere pressure.

we define P , the practical permeability constant, by the following equation:

$$P = \text{GM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} (\text{GM} \cdot \text{l}^{-1})^{-1} \quad \text{XI}$$

where P is the permeability constant and by GM we understand either gram moles or gram ions as the case may be. P has in this equation the dimensions $\text{cm} \cdot \text{sec}^{-1}$, or more properly, $\text{l} \cdot \text{t}^{-1}$, a velocity. This does not differ essentially in principle from many definitions previously offered.

Sometimes the rate of penetration is given only in terms of $\text{GM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$. This is an incomplete characterization. In other words, suppose that three different concentrations of the same substance are used in different experiments. After a certain interval, readings of the concentration of this substance in the cells are taken. It is found that these cells take up the substance from the external solution in an identical fraction of the external concentration. The rate of penetration of the substance from the three solutions was the same, other factors being equal, even though the concentrations in the cell in the different cases were different.

During swelling and shrinking of cells in anisotonic solutions, these processes follow a logarithmic course, like a unimolecular reaction isotherm. They follow this closely enough so that a constant may be calculated in the familiar way, and used as a measure of the rate of water movement. This was shown by LILLIE (1909). The formula given by NORTHRUP (1929) is more correctly derived, but does not give results differing so widely as to justify the additional work of calculation, since in the available physiological data other factors must unavoidably be so inaccurately determined.

The initial and final volumes for example can be calculated quite accurately for spherical cells, such as many echinoderm or annelid eggs; and thus give us information as to the actual amount of water taken in. But in the case of such cells as mammalian erythrocytes, striated muscle cells, or cells of *Spirogyra* the accuracy of such calculations is decidedly less, and in the case of epidermal cells of *Tradescantia*, and various parenchymatous plant cells the calculation can at best only give us an approximation. The calculation of the area of the membrane is subject to the same limitations.

The activity of water within living cells may with reasonable safety be considered initially equal to that in the fluid normally bathing the cell. From this we get the activity gradients across the membrane. For example: mammalian erythrocytes are normally bathed by plasma isotonic with a 1.12% solution of NaCl (PONDER and SASLOW, 1930 b). The osmotic pressure of the solution bathing the erythrocyte was formerly considered to be that of a 1.12% (0.190 M) solution of NaCl, which is 10.0 atmospheres. If we consider the activity coefficient of water, pure, to be 1.0, then its activity as transformed into moles in pure water is 55.5 M, and in a solution whose osmotic pressure is 10.0 atmospheres it will be $55.5 - \frac{10.0}{22.4} \text{ M} = 55.15 \text{ M}$. If we then suspend such erythrocytes in 0.5% NaCl, in which the activity of water, similarly calculated, is 55.34 M, an activity difference of $(55.34 - 55.15) \text{ M} = 0.19 \text{ M}$ will be set up across the plasma membrane of the erythrocyte.

For the purpose of formulation of comparable permeability constants for different substances, such as water, dyes and salts, in this book the activities of these substances is given in terms of molality. In chemical practice the activities are given in terms of a fraction (or multiple) of the activity of the standard state, taken as unity. Thus, in this work, we express the activity of water in the standard state as 55.5 M, rather than as unity.

From data as to initial and final volumes and surface areas, normal (or initial) and final intracellular osmotic pressures, and the velocity constant of the swelling or shrinking process, the permeability to water at the beginning of the process may be calculated using LILLIE's¹⁾ form of the isotherm for the process, e. g.,

$$k = \frac{1}{t} \ln \frac{V_e - V_o}{V_e - V_t} \quad \text{XII}$$

where k is the velocity constant, t , the time, and V_e , V_o , and V_t the volumes of the cell at the end of the process, at the beginning and at time t respectively. If t is in minutes, the change in volume, and therefore the average rate of water intake, during the first minute can be gotten from the expression

¹⁾ LILLIE's equation applies to the case of sea urchin eggs, although its derivation is theoretically unsound.

$$V_t - V_o = \frac{e^k - 1}{e^k} (V_e - V_o) \quad \text{XIII}$$

which is derived as follows:

$$\text{When } t = 1 \quad k = \ln \frac{V_e - V_o}{V_e - V_t} \text{ and } e^k = \frac{V_e - V_o}{V_e - V_t} \quad \text{XIV}$$

$$\text{then} \quad V_e - V_t = \frac{V_e - V_o}{e^k} \quad \text{XV}$$

$$\text{and} \quad V_t = V_e - \frac{V_e - V_o}{e^k} \quad \text{XVI}$$

Subtracting V_o from each side:

$$V_t - V_o = V_e - V_o - \frac{V_e - V_o}{e^k} \quad \text{XVII}$$

$$= \frac{e^k (V_e - V_o) - (V_e - V_o)}{e^k} \quad \text{XVIII}$$

$$= \frac{(e^k - 1)}{e^k} (V_e - V_o) \quad \text{XIX}$$

The permeability, P , may then be calculated:

$$P = \frac{\frac{e^k - 1}{e^k} (V_e - V_o)}{\text{Mol. Vol. of H}_2\text{O}} \cdot \frac{1}{\frac{(\text{surface area of cell})}{(\text{activity difference})}} \quad \text{XX}$$

If volume and area are given in terms of cm^3 and cm^2 , P will represent gram moles per square centimeter per mole per second.

As an example we may cite the results of experiments on the passage of water through collodion membranes: $P \cdot 10^1 = 0.055 - 0.55$ (FUJITA, 1926), 0.102 (COLLANDER, 1926), and 0.055 (NORTHROP, 1929).

These definitions take no account of electrokinetic driving forces, and in some cases, notably for the ions of strong electrolytes, they are inadequate. If electrokinetic forces only were involved permeability might be defined in terms of volts $\cdot \text{cm}^{-1}$ and ζ potential; but activity gradients are never negligible. It is of course obvious that the driving force in such cases is the algebraic sum of the forces due to the activity gradient and the electrokinetic force, and that equilibrium would theoretically be reached when these forces were equal in magnitude and oppositely

directed. But no satisfactory suggestion has been made as to a common unit which might be used to measure the forces due to both activity and potential gradients. In discussing permeability to ions we shall mention possible formulations which might be useful in particular cases if we but knew more about the actual nature and location of the potential gradients. For water and undissociated molecules of weak electrolytes and non-electrolytes the driving force due to activity gradients may be used to obtain a first approximation to the true permeability. For ions, such as the alkali metal ions for example, such calculations are useless as a measure of permeability proper. The questions thus raised are discussed in connection with permeability to the ions of strong electrolytes. It is to be noted carefully here that in trying to evaluate permeability constants we cannot use the thermodynamic work involved in the transfer of molecules or ions from one activity or potential level to another as a measure of the forces acting on the molecules or ions. To the thermodynamic work must be added work to overcome resistance to the flow of material. This is analogous to frictional resistance and may involve viscosity. The energy expended will usually appear as heat. Thus SCHREINEMAKERS' (1930) analysis of the sequence of changes in the composition of solutions separated by membranes "in which forces act" (e. g., electrokinetic forces) does not allow us to calculate permeability constants. A long series of papers by this author describes and classifies osmotic phenomena in a wide variety of systems, but seems to us to multiply terms and concepts needlessly, and to yield little which can be applied to the understanding or quantitative measurement of permeability.

Certain complications remain to be considered. (1) In the first place, one cannot measure directly the differential $\frac{dn}{dt}$, which represents the rate of passage of substance across the membrane. Three ways are open for evading this difficulty: (a) approximate values may be obtained by using increments $\frac{\Delta n}{\Delta t}$, i. e., measuring the amount of substance crossing the membrane in some short interval of time; (b) total amounts of material which have penetrated at various times may be plotted against time, and the differentials determined graphically from the slope of the curve at various times; and (c) the differential equation may be inte-

grated as has been done for unimolecular reactions in Equations II and III on page 7.

(2) This however leads to a second complication in that the area of the membrane may vary as a function of the amount of substance which has passed through the membrane, particularly if water be the diffusing substance. The area then becomes a more or less complicated function of time, of the shape of the cell, etc. Various integrations dealing with this factor in special cases are considered below. A general discussion of the process of diffusion with mathematical treatment is given by JACOBS (1935).

(3) In all of the above discussion it is assumed that diffusion to and from the membrane is relatively so rapid that outside of the membrane there are no appreciable concentration gradients. If this be not so, then at one or both surfaces of the membrane the rate of concentration change will be given by the following equation:

$$\frac{dc}{dt} = \pm k \cdot \frac{da}{dx} \pm k' \cdot \frac{da'}{dx}$$

Here a' and a , k' and k are the activities and diffusion constants within the membrane and in the medium outside the membrane, respectively. If the membrane be regarded as porous, one may assume its permeability to be proportional to the relative area of the pores, and k to be the same in the pores as outside. Then using the equation

$$\left(\frac{\partial c}{\partial t}\right)_x = k \cdot \frac{aa}{dx}$$

(FICK, 1855; our notation) one may proceed as has been done by MIYAZAKI (1827) who has developed an interesting set of equations which were experimentally verified in models where concentration gradients outside the membrane were abolished by stirring. The equations are shown to be applicable to cells of small size and low permeability even in the absence of stirring. The same equations would be applicable to other types of membranes by the use of suitable ratios between the different diffusion constants analogous to MIYAZAKI's ratios of the effective cross sections of the diffusion paths. This development is limited to cases where one of the outside concentrations is essentially constant. In practice large volumes of surrounding fluid or renewal thereof

can be made to provide this essential condition. See also JACOBS (1935).

(4) According to certain hypotheses discussed below the plasma membrane is a thin homogeneous layer of fluid immiscible with water. Here the rate of diffusion through the two interfaces, one after the other, measures the permeability. It will be dependent upon partition coefficients and phase boundary conditions. A detailed analysis of a special case, the "artificial cell" of OSTERHOUT and STANLEY (1932), has been made by LONGSWORTH (1933). In this study no reference is made to a permeability constant as such, it being replaced by a function of the diffusion constant and partition coefficient of the substance in question. This paper will be referred to again in connection with strong electrolytes.

Experiments by CHAMBERS and KOPAC (1937) in which oil droplets have been forced through the plasma membrane without coalescing with it, suggest that the external phase is not completely lipid.

Classes of substances

For the purpose of this book substances may be grouped under five heads: water; non-electrolytes, i. e., substances having dissociation constants less than about 10^{-12} ; weak electrolytes whose dissociation constants lie between 10^{-12} and 10^{-2} ; strong electrolytes having larger dissociation constants, and finally dyes, which belong largely to the weak electrolytes, but are most conveniently treated in a separate chapter. This arbitrary division sets up approximate limits for groups of substances whose permeability relations require separate consideration. The groups intergrade and the limits set cannot and are not strictly adhered to.

Normal and abnormal permeability

From what has been said above it is evident that both activity gradients and permeability must be subject to constant fluctuation during the life of a cell. So long as these fluctuations do not go beyond certain limits, the cell may be said to be in a normal condition, but the exact limits of normality must necessarily be vague; the boundary between normality and injury is difficult to ascertain. Strictly speaking, any experimental method which in any way transgresses the limits of variability commonly experienced by

the cells used renders them abnormal. In practice, we may distinguish between merely abnormal cells which are able to make substantially perfect recoveries after restoration to their normal environment and truly injured cells which do not recover, but instead go on to more or less immediate death. Experiments which inflict irreversible injury on the cells used must be interpreted with great caution and should in no case be considered to invalidate contradictory experiments in which reversible injury or no perceptible injury occurs. JUST (1939) adopts normal development of egg cells as the essential criterion of normality.

CHAPTER II

THE WATER EQUILIBRIUM OF LIVING CELLS. THEORETICAL CONSIDERATIONS

The presence and distribution of water in protoplasm

Water is universally present in living cells, and is apparently indispensable to their continued existence. It usually constitutes from 70 to 95% of the weight of typical tissues, and therefore, an overwhelming mole fraction of the molecules present in protoplasm are water molecules, for nearly all other molecules present are heavier than water molecules. Similarly, we are forced to suppose that water typically exceeds any other substance, in fact all other substances put together, in the volume which it occupies in protoplasm (Table I).

It is, therefore, natural to assume that protoplasm is a system in which fats and fat-soluble materials constitute one phase, while another phase is made up of proteins, salts, sugars, and so on in aqueous solution. We must probably take into account protein and other micelles as additional phases, which may be in part the same as the various microscopically visible granules. This idea is in accord with what we know of the absolute viscosity of the cytoplasmic matrix of many living cells. (See HEILBRUNN 1928). The precise distribution of the phases and of the components within the aqueous phase has been the subject of almost endless speculation, as have also the physical and chemical unions between the different chemical species. Some of these are discussed in connection with theories of protoplasmic structure (see p. 30), and only one need be mentioned in this place. This hypothesis was suggested originally by studies on a system consisting of two phases: "Phenol dissolved in water" and "water dissolved in phenol" (FISCHER 1923). In these studies, the tacit assumption is made that, in each phase, the solute is dispersed discontinuously

in a continuous solvent. This idea was extended to soap-water systems (FISCHER 1924), the gel state being considered to be a "solution" of water in soap. From this point it was simple to extend the idea to protein gels (FISCHER 1924), and from thence to protoplasm (FISCHER 1923). According to this theory, protoplasm is a system in which water is distributed discontinuously in protein or other complex molecules acting as a solvent, the whole being immiscible with water and not bounded by any differentiated surface layer or plasma membrane. A very similar view has been adopted by LEPESCHKIN (1928, 1930).

Table I. The numbers of molecules of different kinds present in a single typical erythrocyte

Water	980000×10^6
Hemoglobin	300×10^6
Phosphatide (calculated as lecithin)	300×10^6
Cholesterol	230×10^6
Glucose	295×10^6
Urea	295×10^6
Adenine-ribose nucleotide	68×10^6
Glutathione	52×10^6
Thioneine	44×10^6
Creatine	26×10^6
Creatinine	8×10^6
Uric acid.	7×10^6
K ⁺	6300×10^6
Cl ⁻	2800×10^6
Mg ⁺⁺	70×10^6
HPO ₄ ⁼	70×10^6
H ⁺	$.0022 \times 10^6$

This point of view will not be adopted here for the following reasons: (a) It is improbable that the phenol-water systems are analogous to the other systems mentioned, since these have been shown by other studies (LAING and MCBAIN 1920, NORTHRUP & KUNITZ 1931) to have a complex micellar structure, with mutual interpenetration of two or more phases; (b) the abundance and small size of water molecules make it, *a priori*, improbable that they would be completely separated into separate phases by the large and complex protein molecules; (c) water-soluble dyes and salts diffuse freely and rapidly within the cell; and (d) the evidence for the existence of a semi-permeable membrane and for

the spontaneous formation under certain conditions on freshly exposed protoplasmic surfaces of a film which prevents free intermixture of cytoplasm and surrounding solution shows that protoplasm without a special surface membrane is freely miscible with water. (HEILBRUNN 1928, COSTELLO 1932, REZNIKOFF and CHAMBERS 1925).

We shall, therefore, assume that water is continuous throughout typical protoplasm.

The driving forces

Electrophoresis. In the case of water electrical forces must be presumed to consist of potential gradients acting upon an electrokinetic potential between water and the membrane material. Either the water forms continuous channels through the membrane (pore theory) or is dispersed in it (lipoid theory as modified by recent workers). The corresponding modes of transport, electroosmosis and cataphoresis, do not differ fundamentally, and to evaluate the force acting in either case we should have to know the electrokinetic potential. In artificial osmotic cells the influence of electrical forces can be studied with some degree of success (BIKERMAN 1935, BRIGGS 1933, TEORELL 1935 and SCHREINEMAKERS 1929, 1933). But in living cells, though we can sometimes (e. g., for erythrocytes) guess at the potential gradient, the electrokinetic potential as well as other essential facts are quite unknown. Therefore, we cannot now evaluate the electrical forces involved in the transport of water in living cells. But from the fact that their osmotic shrinkage and swelling can be approximately predicted upon an activity (osmotic) basis alone it would appear that electrical forces usually play a secondary role.

Activity gradients. Neglecting electrical forces it should follow that when the osmotic pressure of its environment is changed a living cell should gain or lose water enough to make its osmotic pressure again equal to that of the environment. If the aqueous portion of the cell were an ideal dilute solution and no other complications were involved then the volume, V , of the cell and the external osmotic pressure, Π_e , (which equals Π_i , the internal osmotic pressure) should be so related that

$$\Pi_e \cdot V = \text{const.}$$

This simple relationship, often referred to as the BOYLE-MARIOTTE law, is not actually obeyed in most cases. The cause for this non-ideal behaviour belong to two groups; the colligative properties of solutions of the nature and concentration (of the order of 0.1 M) concerned; and structural or functional features of the living cell.

The colligative properties of concentrated solutions. Non-ideality of solutions. As soon as we mix an appreciable amount of one substance with another substance to form a "homogeneous phase", such as a solution, the activities of both substances are altered. When, for example, we add sucrose to water, the vapor pressure of water from the solution is altered, and at the same time, such colligative properties as boiling point, freezing point, and osmotic pressure undergo change. The activity and fugacity of water have been reduced and the osmotic pressure of the solution is proportional to the activity depression, i. e., the difference between the activity of the water in the solution and that of pure water at the same pressure and temperature. In terms of fugacity,

$$\Pi = \frac{RT}{V} \ln \frac{f_0}{f}.$$

This is a result, in the first place, of "diluting" the water with sucrose. Its stoichiometric concentration expressed as the mole fraction (i. e., gram moles of water divided by total gram moles of all the components of the solution) has been lessened¹). In dilute solutions of sucrose, this factor accounts almost quantitatively for the decrease in activity of the water, and the activity varies as the molality of water in the solution. Thus where the molecular weight of water is taken as 18.02,

$$a = m \cdot \frac{1000}{18.02} \cong m \cdot 55.5.$$

Such solutions are termed ideal solutions. But in more concentrated solutions, and in solutions of strong or weak electrolytes, the activity of the water is no longer proportional to the mole fraction of water in the solution. The activity may be increased above that of ideal solutions as a result of the net mutual

¹) Polymer formation does not affect the application of the equation (BARNES 1937).

electrostatic attraction of ions (DEBYE and HÜCKEL 1923), and it will be decreased as a result of attractive forces between water and solute molecules. One or the other of these opposed effects may predominate according to the composition of the solution.

Disorder The first effect will vary with water intake or loss by the cell since an increase in volume will decrease the attractive forces between the ions of strong electrolytes and thus decrease the activity of water. Furthermore, an increase in volume will increase the dissociation of weak electrolytes (organic acids, amino acids, etc.) present in the cell, thus increasing the amount of osmotically active material in the cell, and decreasing the activity of water.

Both strong and weak electrolytes, therefore, respond to loss of water from the cell by so changing the activity of water as to resist the impressed change, thus partially compensating for the loss of water and necessitating still further water loss to make up for the compensation. Conversely osmotic intake of water leads to changes necessitating still further water intake. This is a typical example of the consequences of LE CHATELIER's theorem.

The second effect is important in concentrated solutions.

Laws have been developed for ideal concentrated solutions in which the repulsion between the ions is negligible by comparison with the attraction between solute and solvent molecules (NERNST 1894), but the quantitative aspects need not concern us here. If water be added to a relatively large amount of potential solute, such as gelatin, so as to make a very concentrated "solution" (which may be solid), the same kind of forces come into play, water is attracted to the potential solute with such force that appreciable heat may be liberated. (ROSENBOHM 1914). Such a system can bind and retain water so tenaciously that the vapor pressure of water with which it is in equilibrium is enormously less than that of pure water at the same temperature. The activity even more than the mole fraction of the absorbed water is greatly less than one. This phenomenon is particularly marked in the case of colloidal solutes, such as starch, and is, in such cases, referred to as "imbibition". Essentially, it is the very same process as that which, in the case of a concentrated sugar solution, results in reducing the activity coefficient of the water and giving to the solution an osmotic pressure in excess of that calculated from the mole fractions of water and sugar.

Imbibition and Osmosis. Attempts are frequently made to distinguish between absorption of water by osmosis on the one hand as opposed to hydration or "imbibition" on the other. The value of such a distinction is not clear. Imbibition is simply absorption of water by a system which is in effect an exceedingly concentrated solution, and can often be shown to obey the laws of ideal concentrated solutions referred to above. This has been shown, for example, by KATZ for dried gelatin. (KATZ 1918, but see SPONSLER 1939.)

From this simple point of view such phenomena as CARL LUDWIG's often cited experiment (LUDWIG 1849), in which dried pig's bladder absorbed water from a saturated solution of sodium chloride, causing the latter to crystallize out, is evidently to be explained on the same basis as the absorption of water by the bladder considered as a solid saturated solution, viz: that the activity of water in the dried pig's bladder is less than in the solution. The fugacity of water from saturated solutions of various substances is not identical. No one would be astonished if a concentrated sucrose solution was able to withdraw water from a saturated solution of calcium carbonate, and thereby lead to the precipitation of the latter; and no one would hesitate to refer this phenomenon to osmotic water exchange. LUDWIG's experiment is no more inexplicable by osmotic forces than is the example cited. In both cases water moves along its activity gradient.

Imbibition and osmotic pressure may therefore be distinguished, but not in the traditional way. Imbibition, in any fundamentally sound sense, is not a property of colloidal systems alone, but may be exhibited by homogeneous solutions of crystalloids, among which are such diverse substances as gases, molecules of solid or liquid non-electrolytes, and ions of electrolytes. Details of water absorption by proteins is of great interest in this connection. Theories given by SPONSLER (1939) are discussed on page 28.

It therefore seems best not to use the terms "imbibition" and "osmosis" in the traditional sense, in which they are supposed to refer to intake of water by heterogeneous and homogeneous systems respectively. The possibility of distinguishing experimentally between the intake of water into a living cell as a result solely of a lower mole fraction of water in the cell, and without any superimposed change in the activity of the absorbed water,

as distinguished from the intake of water with subsequent change of its activity will be discussed later.

Hydration. The actual mechanism by which the activity of water is reduced in concentrated solutions is not simple, however. There appear to be several processes, collectively known as hydration, which reduce the activity of water in the solution. One of these is that by which water combines with ferric, cobaltic, or manganic ions for example, and is a molecular bonding in which there occurs a mutual cancellation of magnetic moments (FREED and KASPER 1930). Here we are concerned with a definite, relatively permanent bond between a particular molecule of water and a particular portion of a given ferric ion, or other paramagnetic ion or radical, and the combination is stoichiometric.

Another type of hydration is that which binds water molecules to alkali metal and alkaline earth ions, among others, and is probably also that by which hydration of proteins and many other organic colloids is effected. In this type, hydration is due to the action of static fields upon the water molecules, which, because of their high specific inductive capacity, (see, for example, EUCKEN, JETTE and LA MER, 1925, p. 346) tend more than other molecules to congregate around ions or other sources of electrostatic fields. In the case of ions of the alkali metal and alkaline earth series definite coordination numbers (4, 6, or 8) corresponding to maximum stability of the ion hydrates thus formed may be deduced (GARRICK 1930) but the zone of attraction presumably extends beyond these few water molecules, and as in the case of larger ions, such as proteins, this effect is more probably a general one produced by a static field acting upon an indefinite number of water molecules; furthermore, molecules continually enter and leave the region of attraction¹). It is obvious that so-called "residual" fields, and also fields arising at interfaces by reason of differences in dielectric constants, may act like the fields due to ionization. The effect of electrostatic hydration will be to restrict the freedom of water to move away from the region where the hydration occurs, or, in other words, to reduce the fugacity of the water from that region. This is essentially the

¹) In this connection see BERNAL (1938) and SPONSLER (1939) on hydration of proteins. See page 28.

mechanism of the increase in molal osmotic pressure in concentrated sugar solutions.

In solutions of strong electrolytes as ordinarily encountered in biology, the mutual attractive forces between ions of unlike sign more than compensate for this effect, and create the illusion of quite incomplete dissociation as mentioned above; but in the case of concentrated colloidal gels and sols, the effect of hydration can outweigh the effect of mutual attractions between unlike charges and give rise to an effect externally identical with any other mechanism by which the activity of water is reduced and osmotic pressure created.

A third type of "hydration" is that due in reality to osmotic forces within micelles, which reduces the fugacity of water from a sol, because it reduces the mole fraction of water in the external phase in which the micelles are dispersed. The fugacity of the water from the micelles being, at equilibrium, equal to that from the extra-micellar phase, the fugacity of water from a micelle containing sol or gel as a whole is correspondingly low. It makes no difference whether we consider the micelles or the extra-micellar phase, or both, to control the intake or loss of water by the system. It must be remembered, however, that elastic forces between micelles are also potentially effective in determining water intake or loss from a gel as a whole¹).

Still a fourth effect observed in certain types of gels may serve to lower still further the activity of the free water in the gel. This arises from the fact that in such gels (e. g., silica gels) the fluid lies in capillary spaces and is bounded from the surrounding air by sharply concave menisci. The vapor pressure (p_1) in equilibrium with such a curved surface is less than that (p) in equilibrium with the same solution bounded by relatively flat surfaces by an amount depending upon the radius of the meniscus (r), the surface tension (γ) and the densities of the fluid and vapor (d_1 and d). The relation is

$$p - p_1 = \frac{2\gamma d}{r d_1}.$$

¹) A somewhat similar grouping of different types of hydration is given by G. F. HÜTTIG [Koll. Zeitschr. 58: 44 (1932)] on the basis of x-ray crystallography of gels during dehydration. See also BERNAL (1938) and SPONSLER (1939).

As a result of this a solution occupying the pores of a gel in contact with air (or any gas) will have a lower vapor pressure, and therefore also a lower fugacity and activity than it would have in contact with an aqueous solution or other miscible fluid.

In living systems the first, or magnetic, type of hydration must be the exception because of the scarcity of paramagnetic material, and need not be taken into further consideration here. Only if one studies the water relations of cells exposed to a gas phase (e. g., lung epithelium) would it be necessary to consider the fourth type, i. e., capillary hydration. The second and third types must be of rather frequent occurrence; but they have not yet been distinguished experimentally or theoretically in enough cases to give us any clear idea as to their relative importance. Possibly a better recognition of their distinctive properties would help to clear up conflicting evidence as to the amount of "bound water", so called, in colloidal and living systems. This, because of its very general interest, and the fact that it is water of hydration in some sense, may well be considered briefly at this point.

Bound water in sols, gels and living systems may be defined in various ways, all of which involve ultimately the idea that part of the analytically determinable water in the system is withdrawn in some way from the rest, or "free" water. It is often, but not necessarily, supposed that "bound" water cannot act as a solvent. As pointed out by BRIGGS (1931, 1932) this involves the idea that kinetic energy of water molecules which have become bound is transformed into heat or useful work, and the amount of water so bound must depend upon the activity of the free water. This corresponds to the behavior of the ideal concentrated solution of NERNST (1894). However the results of PARPART and SHULL (1935) in which 50 per cent of cell of the mammalian erythrocyte acts as a solvent for certain non-electrolytes but not for urea, may be interpreted to mean that urea dissolves in the bound water also. The principal types of methods for determining bound water are: (1) Addition of a solute to the system in known proportion to the total water present, and comparison of the resulting additional depression of the freezing point, with that resulting when the solute used is alone in solution. This is usually referred to as the "cryoscopic method". (NEWTON and GORTNER 1922, NEWTON 1924, EGE 1927, GORTNER 1930, SUNDERMANN 1932, GORTNER and GORTNER 1934, MORUZZI

1934). (2) Supercooling with subsequent measurement of the heat of fusion of the free water, the bound water being defined as that remaining unfrozen. (MÜLLER-THURGAU 1880, RUBNER 1922, PLANK 1925, THOENES 1925, ROBINSON 1928, ST. JOHN 1931, HEISS 1933, SMITH 1934, CHRYSLER 1934). This "calorimetric" method is preferred by SAYRE (1932) who compared the first three methods noted here. But see the critique by MENNIE (1932) and HAMPTON and MENNIE (1932). (3) Measurements of the dilation of systems at the freezing temperature (-20°C) as a measure of the amount of "free" water frozen. (MCCOOL and MILLAR 1920, ROSA 1921, MORAN 1930, JONES and GORTNER 1932). Other methods involving freezing have been employed by MORAN (1932, 1934a, 1934b), and by BROOKS (1933, 1934). (4) Measurements of vapor pressure in simple systems of known composition (BRIGGS 1931) or the change in vapor pressure in systems altered as in (1) HILL (1930). (5) Comparison of osmotic volume changes with those predicted if the whole of the analytically determined water were free to act as a normal solvent (OVERTON 1902, EGE 1927). (6) Quantitative determination of the concentrations of diverse reference solutes in the ultrafiltrate from solutions containing both reference solute and the colloidal solute which is to be tested for hydration. (GREENBERG and GREENBERG 1933, AMBARD and DEVILLER 1935). This method, like (1), (4) and (5) assumes that bound water should be incapable of acting as a solvent. Criticism of this method by BULL (1933) has been adequately met by GREENBERG and COHN (1934). GREENBERG's evidence (1933, 1934) tends to disprove the existence of appreciable amounts of bound water in protein and other colloidal sols, and the directness and simplicity of his method strengthen his contention. Other methods of determining "bound" water have been described by NICLOUX (1934a), by ERNST and CZIMMER (1931), NEWTON (1924) and by JOCHIMS (1932). JOCHIMS depends upon the use of a modified form of the EINSTEIN equation relating the viscosity of a sol to the relative volume of the disperse phase. This seems too indirect and unreliable for any except comparative determinations such as JOCHIMS has used it for. It appears in general that methods employing freezing (1, 2, 3) determine the amount of water bound under conditions of temperature at which the activity of free water differs from that at temperatures compatible with normal life. It might also be ob-

jected that the pressure employed in ultrafiltration (6) changes the activity of the free water. Any change in the activity of free water should be reflected in the amount of water bound by unit weight of any given colloid. BRIGGS (1932) points out the theoretical necessity of this relation, and it has been observed experimentally (HAMPTON and MENNIE 1932).

No method so far suggested is entirely above criticism¹), and it is not surprising that there is no general agreement as to the amount and importance of bound water in biological systems. One group of investigators considers that the bound water in living systems and biological fluids is very considerable, and another group considers it almost negligible. In general the former group has studied either plant tissues or juices or very concentrated gels *in vitro*, and the latter has studied blood plasma, blood serum, red blood corpuscles or muscle. It is possible that binding of water is ordinarily important only in plant tissues and systems containing very little water. It is to be noted, however, that many of the earlier workers who claimed to have found large amounts of bound water, assumed that all free water was frozen at -20°C . This is certainly generally untrue (HEISS 1933 b, MORAN 1934 a).

Other sources of confusion may lie in the fact pointed out by MORAN (1930) that unfrozen (and therefore presumably "bound") water in frozen tissues may still act as a solvent for salts and in failure to make adequate allowance for salts and ionized colloids in the systems supposed to "bind" water. Furthermore, "hydration" by osmotic water exchange between micelles and dispersion medium may need to be taken into account. KUNITZ (1926, 1927) has found that only by taking into account this feature of colloidal behavior is it possible to account quantitatively for the characteristics of certain gelatin sols. Although many biologically important proteins form molecular disperse sols, it is still possible that KUNITZ' suggested explanation would help to reconcile the present discordances.

In this connection, the concept of the structure of bound water in protein as developed by SPONSLER (1939) is of interest²).

¹) In this connection consult BULL (1933), BRIGGS (1931, 1932), HAMPTON and MENNIE (1932), GORTNER and GORTNER (1934), GREENBERG and COHN (1934), MORAN (1930), SAYRE (1932), SUNDERMANN (1932).

²) See also BERNAL (1938).

He believes that desiccated protein molecules exposed to water bind the water by hydrogen bonds arising first at the side-chain groups and then at the peptide linkages, followed by further addition to the side-chains which are predominantly carboxyls

and amides. This absorption proceeds in several steps which are actually continuous rather than distinct. This concept is based on experiments on gelatin. Starting with desiccated gelatin, water is added gradually, and at a 15 to 18 per cent water content, the side-chain hydrophilic centers are to a great extent satisfied. The side-chain spacing shows this, with practically no water in the backbone however. At 33% approximate most of the hydration centers of the backbone have become bridged to water and a little more on the side chains. At this stage practically all of the coordination points are satisfied. The backbone spacing has increased and the side-chain spacing has also increased. Further addition of water spreads the side-chain spacing, while up to 90% of water, the backbone

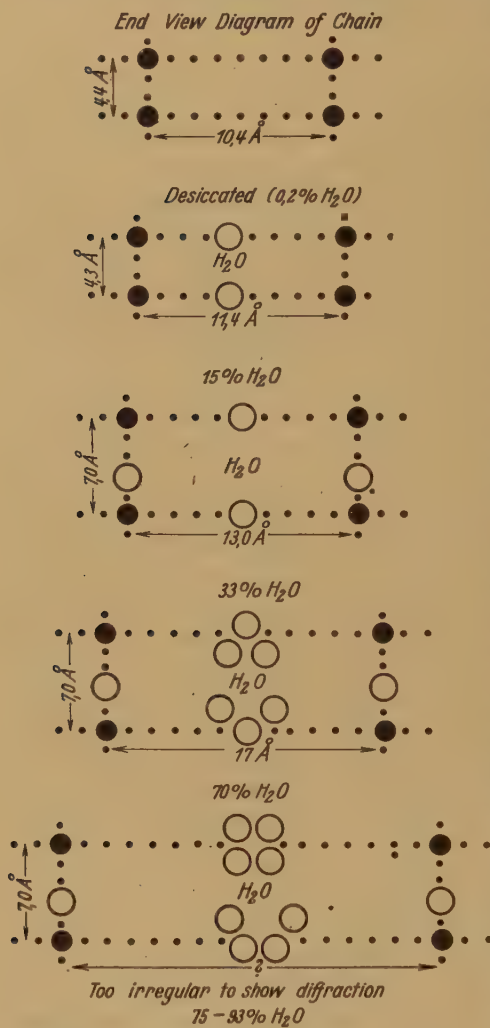


Figure 1. Diagram of backbone and side-chain spacing of a hypothetical molecule with increasing per cent of water.

spacing is still in evidence at the same 7.0 \AA distance as at 33%. The side-chain spacing has increased from 10.4 \AA at 2% to 11.4 at 15%; to 13 \AA at 33%; to 17 \AA at 70%; and is not obtained above about 75% water. The diagram illustrates this idea (SPONSLER 1939). In this figure, x-ray atomic patterns of

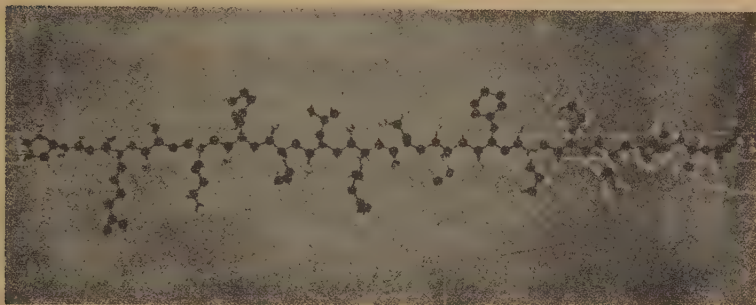


Figure 2. Showing 1/15 of a molecular chain of 16 residues.

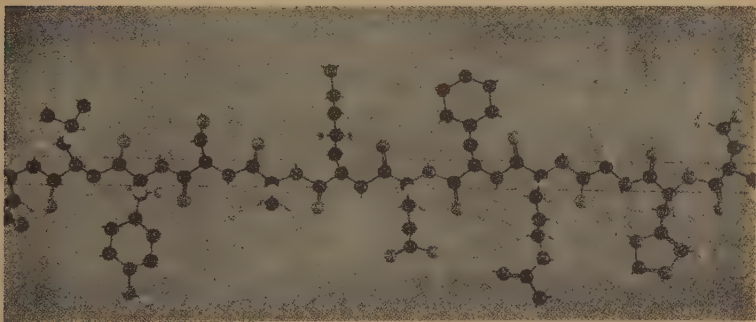


Figure 2A. Showing 1/30 of a protein molecular chain of 288 residues with a M. W. of 38,000.

a hypothetical molecule with increasing per cent of water is shown. The dots and solid circles may be used to represent atoms (C, O, and N); open circles, water molecules, and the distance in two dimensions in \AA units. As more water is added, the distance between the atoms increases (Figure 1). Figure 2 shows the arrangement of molecules and atoms consisting of 1/15 of a protein molecular chain of about 16 residues (SPONSLER 1939).

In the present state of our knowledge of this difficult subject, it seems best to await further experimental evidence before trying to translate known and experimentally measurable activities of water in biological systems into terms of a doubtful real "bound" water. So long as we think in terms of activity and activity gradients of water, we can analyze and interpret experiments with the minimum danger of arriving at illogical conclusions.

Structural or functional features of living cells

Non-solvent volume. Most living cells contain appreciable amounts of material such as fats, sterols, or various kinds of crystals, which do not undergo any measurable change of volume on varying the activity of water in their environment. Obviously such inclusions do not swell or shrink like the rest of a cell when it is placed in hypo- or hypertonic solution. Allowance must therefore be made for them in formulating equations relating to osmotic changes in volume.

The effects of this non-solvent space ("nicht-lösender Raum") on the change in volume of a cell to be expected from a given change in external osmotic pressure was pointed out by HAMBURGER (1898), although the term "non-solvent space" was of later origin. HAMBURGER placed red blood cells in hypo- and hypertonic solutions and measured the resulting volumes by HEDIN's hematocrit method (HEDIN 1891). This method consists in centrifuging at the same speed and for the same length of time the cells of equal quantities of the original blood suspensions, and noting the relative volumes of sediment.

HAMBURGER found that red blood cells transferred from 0.9% to 1.5% NaCl became only 17.5% smaller. If their volumes were inversely proportional to the external osmotic pressure, they should have become 40% smaller, but if a certain volume b in the cells were occupied by material not changing in volume with change in osmotic pressure then the following relation should hold:

$$\Pi_0 (V_0 - b) = \Pi (V_1 - b)$$

where Π_0 , Π , V_0 , V_1 represent the initial and final osmotic pressures and volumes respectively. Such calculations led HAMBURGER (1898) to conclude that in mammalian erythrocytes 50—55% of the volume "aus einem festen Gerüst (Protoplasma) besteht, zwischen welchem die intracelluläre Flüssigkeit vertheilt

ist". It is probable that HAMBURGER meant to include the hemoglobin as well as the stroma in this non-solvent space. The non-solvent space of particular cells will be discussed in connection with the water equilibrium relations of the different cells (Chapter III).

POLANYI (1920) who originated the expression "nicht-lösender Raum", compared the conductivity of serum or casein solution with that of a salt solution brought into diffusion equilibrium with it through a protein-impermeable membrane. The lower conductivity of the former was attributed to the fact that a part of their volume was made up of relatively non-conducting protein material. In view of the probable partial ionization of the proteins and the operation of membrane equilibrium this can only be regarded as yielding a rough approximation to the actual volume of the disperse phase which presumably consists of protein micelles. The experiments show, however, that non-solvent volume must be considered to include dispersed proteins as well as non-aqueous materials such as fats, lipins, sterols, etc. It will be recognized that this type of non-solvent volume is analogous to the term b in VAN DER WAALS' equation of state for gases which is

$$\left(P + \frac{a}{V^2}\right)(V - b) = RT$$

To the actual volume occupied by proteins, lipins, and other colloids must also be added the volume occupied by their water of hydration, as shown by KUNITZ (1926, 1927). Since the amount of this water varies with the activity of water in the system, swelling and shrinkage of the cell will be further complicated by concurrent changes in non-solvent volume. Protoplasm, with its rather high protein content, is certainly a system likely to show marked effects of non-solvent volume in its responses to change in external osmotic pressure.

A summary has been made in Table II to show the effects of correction for non-solvent on solute movement.

The remaining causes of deviation are (b) physical, (c) biochemical and (d) consequences of the structure of the living cells. These affect the activity or the activity coefficient of the water within living or artificial cells, and will be taken up in detail in the following paragraphs.

Physical factors affecting the activity of water in living cells. Two factors are potentially important: temperature and pressure, i. e., hydrostatic pressure. The temperature difference between a living cell and the fluid bathing it will always be so small that its effect on osmotic equilibrium, expressed by the equation $\Pi = \frac{RT}{V}$, will be quite negligible by comparison with other sources of error, unless it leads to shifts in chemical equilibria within the cell (JACOBS and PARPART 1931). (See Chapter III). Not so in the case of pressure. The fugacity of a pure liquid, as measured by its own vapor pressure, increases as the external pressure increases. The relation between f , the fugacity, and P , the pressure, being approximately

$$RT \ln \frac{f_1}{f_2} = \bar{V} (P_1 - P_2)$$

where \bar{V} , the partial molal volume, may be regarded as constant. (LEWIS and RANDALL 1923). This same change in fugacity occurs when a solution is subjected to pressure so that solutions of identical chemical composition will have different osmotic pressures if they are under different external pressures.

The effect of pressure on the fugacity of the solutes is very small in dilute solutions; furthermore, so long as we are dealing with a strictly semi-permeable membrane, the fugacity of the solutes has no bearing on the osmotic equilibrium. It may therefore be neglected in the present connection.

Living protoplasm is nearly always under more than the pressure of the surrounding fluid, because of the inwardly directed force due to surface tension, or to the elastic resistance to distention displayed by the protoplasm itself, or to the cell wall, or other surrounding tissues. All of these will tend to increase the fugacity of the protoplasmic water.

Turgor pressure and suction force. This effect is familiar to botanists under the name of "turgor pressure", which in common with all types of external pressure has the effect of making the actual osmotic pressure smaller than that which would be calculated from chemical composition alone, assuming atmospheric pressure.

For example, let us suppose the turgor of a plant cell to result from the presence of solutes in its sap; and that when this

sap is extracted from the cell it is found by physicochemical methods to have an osmotic pressure of ten atmospheres. When such a cell is in equilibrium with distilled water, the elastic cell wall which has been stretched presses back against the protoplasm, thereby subjecting it to a hydrostatic pressure great enough to increase the fugacity of water from the sap to a value equal to its fugacity from distilled water at the same temperature. Only then will osmosis of water not occur since only then will its tendency to pass out of distilled water into the sap be equal to its tendency to pass from the sap into distilled water. In other words, an internal hydrostatic pressure of ten atmospheres will just suffice to compensate for the presence of the osmotically active solutes in this particular sap.

This, which is really a case of the direct determination of osmotic pressure, also shows how the concepts of fugacity and activity unite supposedly diverse osmotic phenomena into a simple and easily comprehensible system.

The term "suction force" much in vogue among botanists at the present time is obviously only the difference in fugacity between protoplasmic water and external solution; the fugacity in each case being measured at its own proper temperature and (hydrostatic) pressure.

Under biochemical changes may be included those processes commonly grouped under the names of "anatonosis" and "katatonosis". By anatonosis we understand an increase of osmotic pressure inside a living cell as a result of some physical or chemical stimulus. Katatonosis is a decrease of osmotic pressure due to similar causes. Such changes are not well understood. They probably include simple disturbances of equilibria, but not necessarily involving ions as end products. Besides such simple cases there are probably complex series of processes, possibly involving catalysts, and all leading ultimately to an increase or decrease in the total amount of osmotically active material in the cell.

Ana- and katatonic changes are essential mechanisms for the rapid regulation of cell turgor which, in the case of plants at least, is essential to continued existence. Plants depend upon turgor for the support of leaves, flowers and young stems, which must be kept properly oriented and displayed.

Katatonic changes have been shown by VAN RYSSÉLBERGHE (1898) to occur in hypotonic solutions. In this case calcium oxalate appears in the cell sap in crystalline form, and its ions are thus no longer osmotically active. In this way the amount of osmotically active substance in the cell is decreased. This lowers the intracellular osmotic pressure, and reduces the amount of water which ultimately enters. In this experiment the cells would not obey the BOYLE-MARIOTTE law ($PIV = \text{const.}$), even after due allowance was made for the effect of increased turgor pressure on the actual osmotic pressure; because this law presupposes that the total amount of solute in the cell remains constant.

Anatonosis has been studied by ILIIN (1923, 1924). Sections of leaves of *Rumex acetosa* were placed in various dilute solutions, and at intervals of several hours tests were made to determine the concentration of maltose required to plasmolyse the guard cells. Alkali metal salts caused a progressive increase in this concentration while non-electrolytes did not. Disappearance of starch granules was correlated with increase in plasmolysing concentration. It is therefore concluded that under certain conditions, starch is caused to break down forming soluble and therefore osmotically active substances such as glucose or oxalic acid, and thus to give rise to anatonosis. These findings are in the main confirmed by ARENDS (1925) and by STRUGGER and WEBER (1925). Although alternative interpretations are possible it seems probable that anatonosis does occur fairly frequently, at least in plant cells.

Cells which actually swell or shrink in strict obedience to osmotic forces may also appear not to do so because of imperfect semi-permeability of the plasma membrane.

Any experiment involving a change in the activity gradient of water across the plasma membrane will also change the gradients of all other solutes. Only if these undergo no further appreciable change during the subsequent period of the experiment will the resulting volume change be a result of the supposed osmotic water exchange alone. Although there are many solutes to which cells are very slightly permeable, yet the possibility that solutes as well as water are entering or leaving the cell during an osmotic experiment can never be entirely neglected.

Summary

We thus see that the water exchanges of living cells are not simple. They are determined primarily by activity gradients of water, and secondarily by electrical forces whose effects are as yet poorly evaluated. The activity of water in cells cannot be calculated from the analytically determined mole fraction of water present, since it may be complicated by hydration, ion effects and hydrostatic pressure. Furthermore, the amount of osmotically active material in a cell may be changed by exchange with the external solution or by internal changes in dissociation or metabolic processes.

In the next chapter we shall attempt to show with what degree of accuracy various types of living cells obey the BOYLE-MARIOTTE law ($IV = \text{const.}$), and where material deviations from this law occur shall endeavor to point out the parts played by the different complicating factors.

•CHAPTER III

OSMOTIC RELATIONS OF ERYTHROCYTES

In studying the equilibrium distribution of water in living cells we are immediately faced with the fact that most cells consist of microscopically distinguishable portions whose water content may vary each independently of the other. The most important such parts are the nucleus and the vacuoles; in addition a cell wall may be present and may itself swell or shrink in volume, or may merely add to the complexity of the cell's behavior by its imperfectly elastic resistance to distention. Some cells lack one or more of these complicating factors. Mammalian erythrocytes, since they have no nucleus, no vacuoles, and no cell wall are perhaps from this point of view the simplest living cell known. They have also been more extensively and carefully studied than almost any other living cells. We shall therefore consider first the distribution of water between mammalian erythrocytes and their surrounding medium.

Osmotic equilibria of mammalian erythrocytes. Mammalian erythrocytes are often considered to be moribund. It has even been suggested that they are not really alive. But their rate of oxygen consumption is comparable with that of reptilian and avian erythrocytes which are nucleated and not greatly less than that of normal resting animal tissues in general. The normal rates of oxygen consumption by the erythrocytes of man, rabbit, chicken, and land turtle (*Pseudenys elegans*) fall within the range of 8 to $40 \text{ mm}^3 \text{ gm}^{-1} \text{ hr}^{-1}$ (RAMSEY and WARREN 1930, 1932), while the oxygen consumption of resting frog muscle in oxygen is about 250 to $750 \text{ mm}^3 \text{ gm}^{-1} \text{ hr}^{-1}$ (MEYERHOF 1919). Furthermore, the plasma membrane of erythrocytes appears to possess a semipermeability like that characteristic of living cells, as will appear below. For these and other reasons they must be considered to be living cells, much simplified but not differing in principle from the generality of other living cells.

The chemical and structural composition of mammalian erythrocytes has been discussed in detail by PONDER (1934) and need not be considered at this point.

The earlier work on the water distribution between erythrocytes and suspending medium grew out of studies of the maximal concentrations of salts and other organic and inorganic substances (HAMBURGER 1883, 1886, 1902) just dilute enough to produce traces of hypotonic hemolysis. Such solutions were considered to be isotonic with each other, and deductions were made as to the physical chemistry of the different solutions, following the ideas of DE VRIES (1882, 1884) on isotonic coefficients as deduced from the results of his experiments on plasmolysis. Much more exact data on the relation between erythrocyte volume and external osmotic pressure are available in more recent work by other methods, notably the hematocrit method of HEDIN (1891) and the diffractometric method, originally suggested by YOUNG (1813) and perfected by PONDER and SASLOW (1931).

The hematocrit method consists in the centrifugation of a suspension of erythrocytes in a specially designed tube in which the volume occupied by the sediment of cells can be very accurately determined. If the same amount of blood (or of a uniform suspension of erythrocytes) be mixed with each of several different solutions and equal volumes of each such mixture subjected to hematocrit test under identical conditions of precipitating force and time of centrifugation, then the relative volume of sediment found in different tubes may be used as a measure of the mean relative volume of the individual erythrocytes in the different solutions. Unfortunately various complications detract from the accuracy of this method in practice, and make it necessary to interpret small observed differences in volume with great caution. Such factors include small differences in the specific gravity of the erythrocytes and suspending media, in the surface electric charge (ζ potential) of the erythrocytes, or in their shape, or in the degree to which mutual clumping may affect the rate of sedimentation and consequent proportion of intercellular solution in the sediment. The rigidity of the cells may also affect the completeness with which the suspending medium is squeezed out from between them. It might appear that if KOEPPPE's (1905) suggestion were followed, and centrifugation continued until all the intercellular fluid was pressed out, and the sediment consequently

translucent, then the volume of sediment would be truly proportional to the mean volume of the individual erythrocytes. Or one might adopt the method suggested by MILLAR (1925), who centrifuged samples of blood in hematocrit tubes until the sediment reached a constant volume. This was done at different speeds of centrifugation, and the asymptote of the curve relating the volume of sediment to the speed of centrifugation determined approximately by graphic extrapolation (Fig. 3). The volume thus determined was assumed to be the true cell volume.

But high precipitating forces tend to force water out of the cells, and so reduce the volume of sediment. PONDER and SASLOW

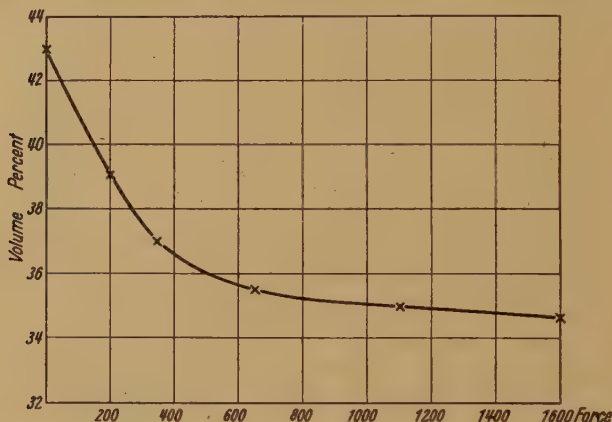


Figure 3. Volume of sediment of erythrocytes to speed of centrifugation.

(1930a) find the erythrocyte volume determined by the hematocrit method, using either translucency (KOEPE) or constant volume (MILLAR) at the end point, to be considerably less than those found by the use of a colorimetric method which these authors regarded as the most accurate method then available.

Since then PONDER and co-workers have elaborated the diffractometric method to a point where it seems preferable to any other. The method depends upon the fact that the diameter of small opaque discs or spheres determines, like a grating, an interference pattern which may be made to appear as a set of light and dark rings (or spectra if the light be not monochromatic). The mean diameter of the cells may be calculated from the diameters of the rings, or more properly by the angle between the

optical axis and the locus of any given maximum or minimum light intensity. For details the original paper by PONDER and SASLOW (1931) should be consulted.

Other methods than these two, viz., diffractometric and hematocrit, have not found extensive application to the problem in hand. Critical discussion of other methods will be found in PONDER (1934). (See EGE 1920, STEWART 1924, BROOKS 1925, PONDER and SASLOW 1930a, MACLEOD and PONDER 1933). SHAPIRO (1935) and JOHNSON and HARVEY (1937) show the effects on volume changes by centrifuging sea urchin eggs and bacteria respectively at high speeds.

In addition to the difficulty of determining the actual relative volume of erythrocytes in a given volume of fluid, there are difficulties arising from actual changes in cell volume as a result of inadequately controlled experimental conditions: pH, (which may be altered by loss or absorption of CO_2 and by other influences), temperature, and oxygenation. These have been critically evaluated and explained by JACOBS and PARPART (1931).

Hypotonic hemolysis and changes in volume of erythrocytes in hypo- and hypertonic salt solutions were described by DONDERS and MOLESCHOTT (1848). KOEPPE (1895) showed that in solutions of sucrose and of different salts erythrocytes assumed volumes which were within the limits of error of his experiments, determined exclusively by osmotic pressure of the suspending fluid. But the first really quantitative studies of the volume changes undergone by erythrocytes in response to known changes in osmotic pressure were made by HAMBURGER (1898) using essentially the hematocrit method of HEDIN (l. c.). Several types of cells were studied including horse erythrocytes. The volume of these cells was found to vary not inversely as the concentration of the solution in which they were suspended, but much less; that is, instead of the equation $CV = \text{constant}$ it was found necessary to use an equation of the type: $C(V - b) = \text{constant}$. Here b represents the non-solvent volume. If V_1 and V_2 are the observed volumes of a sediment when C_1 and C_2 are the corresponding concentrations of the suspending medium, then b is given by the equation:

$$b = \frac{C_1 V_1 - C_2 V_2}{C_1 - C_2}$$

This equation assumes first that the cell, aside from the osmotically inert non-solvent volume acts like a perfect osmometer, and second that osmotic pressure is proportional to concentration both in this "intracellular fluid", as HAMBURGER calls it, and in the suspending medium. KOEPPE (1899) criticizes HAMBURGER's work quite justifiably in that the latter neglected sources of error and wide discrepancies in the non-solvent volumes deduced by the use of different solutes, e. g., in a series of experiments with a single sample of blood, the non-solvent volume calculated from experiments in sucrose solutions is about 38%; and in Na_2SO_4 , 70%; in another series, sucrose, 55%, and in NaCl , 61%. There is also a distinct trend of the non-solvent volume to lower values as the concentration of the suspending fluid increases. KOEPPE attributed these discrepancies to three factors: the change in molal osmotic pressure with concentration, permeability of the erythrocytes to ions, and elasticity of the external surface of the cell. KOEPPE supported conclusions by experiments described in a later paper (KOEPPE 1900).

After a preliminary study of the hematocrit method itself (EGE 1920) and a study of the hematocrit slow volume changes („Nachschwellung“) occurring in some salt solutions EGE (1921) was able to select experimental conditions under which it was possible to evaluate the effects of osmotic pressure on erythrocyte volume more accurately than had been done by previous workers. In a following paper (EGE 1922a) he determined the non-solvent volume of bovine erythrocytes in three independent ways; first by determining the dry weight of a given original mass of erythrocytes; second, by grinding erythrocytes with sand, mixing the resulting material with infusorial earth, and pressing out the contained fluid. In this fluid, assumed to be the same as that normally constituting the bulk of the erythrocytes, he determined the non-solvent volume by the added solute-cryoscopic method (Method 1). The non-solvent volumes determined in this way for a given blood were 28 and 30% respectively. As a third method EGE studied the osmotic volume changes of oxalated rabbit erythrocytes with a non-solvent volume of 35.3% (as determined from the dry weight). In different concentrations of sucrose ($\Delta = -0.272$ to -0.816°) these assumed total volumes by hematocrit agree best with an assumed non-solvent volume of about 40%. This seems to imply that some of the water is

"bound", viz., about 7%, leaving 93% of the total water "free".

discussion
HILL (1930) by the vapor pressure method found that on adding NaCl, KCl, or sucrose to the solution bathing red blood cells whose total water content was 70.2—71.1%, water came out of the cells in amounts corresponding to ratios of free water/total water of 0.955, 0.97, or 0.94 respectively. According to this about 3 to 6% of the water was bound. The addition of water alone yields a similar result: about 5% being found to be "bound". This agrees fairly well with EGE's value, viz., 7%. However, if any of these solutes penetrated into the erythrocytes, less water would have been withdrawn from them and the amount of "bound" water would have appeared to be higher than it really was. It is not certain that any water was in a "bound" condition. A further source of uncertainty lies in the fact that HILL's method assumes vapor pressure to be proportional to rate of evaporation other things being equal. Since surface conditions affect rate of evaporation¹⁾ it is to be suspected, especially in the case of living cells and cell fluids, that this assumption is unwarranted. The agreement between EGE's and HILL's conclusions loses thereby much of its importance.

Moreover there has arisen a good deal of question as to the volumes assumed by erythrocytes in solutions of differing osmotic pressures. This is particularly true in the hypertonic range, i. e., in solutions where the activity of water is less than 0.99 ($\Delta \cong 1.0^\circ \text{C}$) and in quite hypotonic solutions ($\Delta < 0.2^\circ$; $a_{\text{H}_2\text{O}} > 0.999+$). Between these two ranges erythrocytes appear to behave essentially like "perfect osmometers" so long as they remain in media consisting largely of serum or plasma, and so long as they have not been subjected to the action of oxalates. (PONDER and ROBINSON 1934). Presumably some other anticoagulants, such as fluorides and citrates, would act in this respect like oxalates; they have not yet been studied. Cells are said to act like perfect osmometers when after due allowance for nonsolvent volume, as determined by analysis, they obey the equation: $\Pi_e (V - b) = \text{constant}$. This means that little or no water appears to be bound. PONDER and ROBINSON (1934) found that rabbit erythrocytes from defibrinated blood underwent changes of volume in

¹⁾ See for example: BARTLETT, R., and T. C. POULTER, Proc. Iowa Acad. Sci. 34: 214—15 (1927).

diluted serum which corresponded to a "bound" water content of about 5—10%. This agrees with the values found by EGE (1921) and HILL (1930) and may, we believe, be accepted as proved for normal erythrocytes.

The abnormality of erythrocytes from oxalated blood, or suspended in hypotonic salt or glucose solutions is believed by PONDER and SASLOW (1931) to be due to abnormal permeability to solutes. This will be discussed in connection with the solutes concerned. The explanation of EGE's „Nachschwellung“, of different apparent non-solvent volumes in different solution such as NaCl, Na₂SO₄, and glucose observed by KOEPPE and HAMBURGER (See above) and by PONDER and SASLOW (1931), the different responses of the cells of different species to different salt solutions observed by ASHBY (1924) must all be reconsidered in the light of this abnormal condition. In this connection it should be noted that BROOKS (1925) using erythrocytes from defibrinated blood could not duplicate ASHBY's results obtained with citrated blood.

Erythrocytes placed in very hypotonic solutions like those from oxalated blood have large apparent non-solvent volumes, i. e., as much as half of the water they contain appears to be "bound" or osmotically inactive. The reason for this is probably also abnormal permeability with loss of solutes from the cell during swelling. In any event there is no reason to believe that any but osmotic forces are active.

In strongly hypertonic solutions the volume changes of erythrocytes are not so easy to understand. SCHIÖDT (1931) using data published by EGE (1922) showed that EGE as well as KREISKY found erythrocyte volumes in strongly hypertonic solutions from which inconstant and very high non-solvent volumes would be deducible, whereas under more normal osmotic pressure conditions, constant values of non-solvent volume not exceeding 45% are indicated. Furthermore, the exceedingly careful experiments of E. WARBURG (1922) include calculations of the effects of external pH on the volume of erythrocytes which are based upon Ege's estimates of non-solvent volume and agree well with observation. TAKEI (1921) using solutions from hypotonic to very hypertonic ($\Delta = 0.238$ to 6.0° ; $a_{H_2O} = 0.917 - 0.745$) reported ideal osmotic behavior up to a Δ of about 2.0° (varying a little with the solute used), but that higher osmotic

pressures induced swelling. EGE (1922b) justly criticized TAKEI's work, particularly because such concentrated solutions are so dense as to impede centrifugation, and make the volumes found by the hematocrit method too large. TAKEI gave no evidence that centrifugation was complete. Nevertheless, more recent work by PONDER and SASLOW (1930b), employing heparinized blood and the colorimetric method, suggests that there may be a real basis for TAKEI's findings in that under some conditions erythrocytes may fail to shrink in hypertonic salt or glucose solutions. It is a question whether such cells are normal.

GOUGH (1924) using a narrow range of hypertonic NaCl solutions (2.25 — 3.45%; $\Delta = 1.36^\circ - 1.99^\circ$; $a_{H_2O} = 0.722 - 0.705$) found very little shrinkage of sheep and human erythrocytes, and calculated that the volume of the "dispersed phase" must be 65 — 70% of that of the erythrocyte. According to this at least half of the water in the cell would have to be considered to be bound. EGE (1927) criticized this work in much the same way as he did TAKEI's (EGE 1922b). KREVISKY (1930) introduced the term "volume of water osmotically transferable",¹⁾ and believed this to be about 45% for cells in defibrinated blood plus hypertonic NaCl solution, and about 33% for cells washed in 0.85% NaCl solution. It is impossible to tell from KREVISKY's paper what the final osmotic pressures were, and the paper is unclear in other important respects. Nevertheless, it appears to support GOUGH's findings.

It seems probable that Ege is right insofar as normal erythrocytes are concerned and that experimental errors, abnormal permeability or other abnormalities account for the results obtained by TAKEI, GOUGH, and KREVISKY.

The part played by other than osmotic forces in determining the water equilibria of normal erythrocytes seems on the whole to be negligible. Yet there is some evidence for the existence of

¹⁾ We consider this term very undesirable in that it seems to imply that part of the water in the cell would not be removed by any osmotic pressure however high. Certainly no one would doubt that cells placed in 98% ethyl alcohol where $a_{H_2O} = 0.11$ would lose most of their water, and such a process is, as pointed out in Chapter II, an osmotic process. Better terms are available if one interprets "osmotically transferable" water as that actually transferred at a given external osmotic pressure. The term "osmotically transferable" should be discarded.

such forces. WARBURG (1922) for example, has shown that a potential difference across the plasma membrane of the erythrocyte must exist. WARBURG was the first to study the distribution of ions and water between erythrocytes and serum on the basis of the variable base-binding power of the hemoglobin and the serum proteins as factors in a Donnan equilibrium. From his data on the Donnan ratio he calculates the potential difference between the serum and the interior of the erythrocyte under various conditions of CO_2 tension (Table II). The cell content is normally negative to the surrounding medium. Although this is not the electrokinetic potential responsible for the cataphoretic behavior of the erythrocytes as WARBURG assumes, yet it would set up electroosmotic forces which, if the material of the surface

Table II. Showing potential difference between serum and cells at different pH values, calculated according to the HENDERSON-HASSELBACH equation. After E. J. WARBURG 1922.

pH		Potential difference (volts)
Serum	Cells	(S)—(C)
6.49	6.50	—0.006
6.80	6.78	+0.0012
7.00	6.96	+0.0023
7.20	7.13	+0.0041
7.40	7.29	+0.0064
7.60	7.45	+0.0087
7.80	7.60	+0.0116

of the erythrocyte has a positive electrokinetic potential as seems probable, would tend to force water to flow outwards, and give the cell a lower osmotic pressure than the surrounding fluid. A potential difference, such as that of 6.4 m. v. at pH 7.4, if referred to the probable thickness of the erythrocyte cell membrane, about 160 Å (SCHMITT and WAUGH 1939), would necessarily yield a considerable potential gradient, of the order of 10,000 volts cm^{-1} , and if the electrokinetic potential were appreciable, might well lead to a considerable difference in osmotic pressure which should be demonstrable as a difference in the colligative properties, such as vapor pressure or freezing point, between the extracted intracellular fluids of erythrocytes and the surrounding

medium. A somewhat similar conclusion is suggested by KONIKOV's work. KONIKOV (1928) has studied collodion sacs filled with hemoglobin and surrounded by serum albumen solution. By analogy with this model, which is supposed to simulate an erythrocyte, KONIKOV proposes that during specific hemolysis electroosmosis occurs and forces water into the erythrocyte, leading to hemolysis. Although this does not refer directly to the normal state of the cells the model suggests mechanisms which might operate to maintain differences between intra- and extra-cellular osmotic pressure.

Slight differences in osmotic pressure between erythrocytes and serum have been thought by some workers to be normally maintained. HAMBURGER (1897) believed that a difference in freezing point could be demonstrated. Ege (1921b) was inclined to attribute the biconcave shape of these cells to a deficit of osmotic pressure. He presents in confirmation two direct determinations of the osmotic pressures of erythrocyte extracts and corresponding plasmas. His data are supported by MOORE and ROAF (1908) and by others, especially COLLIP (1920) who gives data on the blood of eight species in all, of which the freezing point depression of the serum of defibrinated blood exceeded that of the cell sediment by from 0.019 to 0.075°, the mean difference being 0.043°. The great variation even within a single species is very striking, and is just what might be expected if freezing-point determinations on cell sediments were subject to errors like those found by GROLLMAN (1931) for egg-yolk. Other possible sources of error will suggest themselves. SKUJIN (1927) also claims to have shown that the osmotic pressure within erythrocytes is slightly less than that in sodium chloride solutions (originally 0.0055 — 0.366 M) with which they are in equilibrium.

From the observed changes in Cl in the suspending medium, which are plotted as ordinates in the diagram (Fig. 4) he concludes that when the Cl concentration in the surrounding medium is increased, less Cl enters the cells than would be calculated on the basis of uniform distribution. Unfortunately so many errors and omissions occur in this author's Table II and the accompanying text as to make it impossible to understand his data or to be sure of his meaning. In any event he has completely neglected to take into account the effects of non-solvent volume, of DONNAN equilibrium relationships (cf. below), or of the possibility that

rapid osmotic changes may be followed by slow redistribution of Cl and other ions. SKUJIN's work cannot be considered to contribute any proof of a normally existent difference between intra- and extracellular osmotic pressures.

On the other hand there is a good deal of evidence against the normal existence of analytically detectible osmotic differences between erythrocytes and the surrounding fluid. GRYNs (1896) was not able to find any difference in freezing point between the surrounding and intracellular fluids of erythrocytes. MUKAI (1921) using BARGER's method (1904), which depends essentially upon vapor pressure, was unable to confirm HAMBURGER's conclusions (HAMBURGER 1897). HILL (1930b) using his very

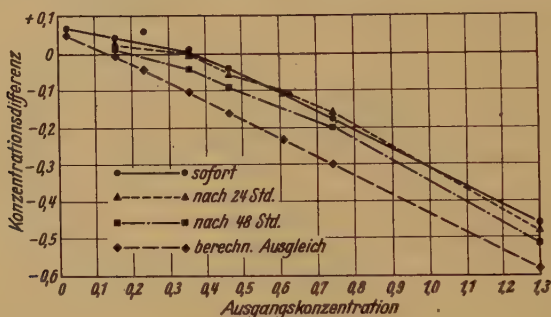


Figure 4. Changes in Cl concentration of the surrounding medium as affecting intake of Cl by cells.

delicate method (HILL 1930a) was also unable to detect any difference in the fugacity of water from defibrinated blood as compared with its laked erythrocytes.

Although the kinetic nature of HILL's method renders this evidence inconclusive, there is a good deal of analytical evidence which tends to support it. (MUKAI 1921; WARBURG 1922; VAN SLYKE, WU and MACLEAN 1923; VAN SLYKE, HASTINGS, MURRAY and SENDROY 1925; WU 1926; see also VAN SLYKE 1926.) This work involves direct determinations of water, hemoglobin, proteins, base, chloride, bicarbonate and pH in mammalian erythrocytes and serum. The results seem to show that the ratio of the total of osmotically active molecules and ions to water is the same in cells and serum (Table III). The question arises whether analyses are exact enough to show, e. g., 5% bound water. Sub-

sequent work has revealed several needed revisions of the assumptions used in these papers; these concern the molecular weight and osmotic pressure of hemoglobin in the cells (this difficulty

Table III. Ratio $\frac{\text{Mols Solute}}{\text{Water}}$ in horse erythrocytes and serum at different CO_2 tensions. (After VAN SLYKES, WU and MACLEAN 1923)

Blood No.	pH of serum	Serum Base + Cl + HCO_3 mille M per kgm H_2O	Cells Base + Cl + HCO_3 + Hemoglobin mille M per kgm H_2O
1	7.66	289	272
	7.11	299	294
2	7.71	297	285
	7.42	307	292
	7.28	314	297
	7.11	318	299
3	7.75	286	285
	7.42	294	294
	7.08	303	303
4	7.69	290	289
	7.35	297	294
	7.06	302	303

was recognized by VAN SLYKE, et al., but could not then be evaluated), the presence of undissociated carbamates in equilibrium with the carbonate system (STADIE and O'BRIEN 1936), and the fairly wide departure of the activities of many components from unity.

KING and SCOTT (1924) have described experiments which are difficult to reconcile with the assumption that the distribution of water is determined by osmotic forces only. They report in effect, that bovine erythrocytes placed in contact with diluted serum take up more water than would be expected if the added water were evenly distributed between erythrocytes and serums. In fact, in some experiments the erythrocytes not only take from the serum all the added water but some more besides. Meanwhile, the erythrocytes themselves increase in volume more than has usually been observed in the case of corresponding dilutions of glucose or salt solutions. The observations

are so inexplicable on the basis of any simple physical relations that it seems best to await detailed confirmation before modifying well established deductions.

A survey of all the factors reveals no convincing evidence for significant effects of other than osmotic forces in determining the distribution of water between mammalian erythrocytes and their surrounding medium. The evidence from the osmotic volume changes of these cells appears to constitute reasonably complete proof that only osmotic driving forces need to be considered in the present state of our knowledge.

The effects of pH, temperature and oxygenation on the volume of erythrocytes serves to confirm the conclusion that these cells are osmotic systems. The analysis in terms of the Donnan membrane equilibrium as made by WARBURG and by VAN SLYKE and his co-workers concerns the effects of pH changes secondary to changes in CO_2 tension. The quantitative formulation is given in Chapter XII in connection with the problem of distribution of ions. (See also DILL 1928). In these papers the experiments reported concern relatively dense suspensions of cells in serum or plasma. JACOBS and PARPART (1931) have modified the equations to adapt them to dilute suspensions, and by the use of improved experimental methods (JACOBS 1930), have studied the effects of pH, temperature, and oxygenation. They were found to produce volume changes quantitatively predictable from the osmotic relations deduced by JACOBS along the lines laid down by WARBURG (See Table IV) and by VAN SLYKE, et al. MACLEOD and PONDER's criticism (1933) of the validity

Table IV. The relative volumes of horse erythrocytes under various pH conditions produced by different CO_2 tensions.

(After E. J. WARBURG 1922)

pH of serum	Relative cell volume:	
	Observed	Calculated
6.5	(100)	(100)
6.8	95.2	97
7.0	92.7	94
7.2	90.7	91
7.4	89.1	90
7.6	87.8	88
7.8	86.3	86

of one basic assumption made in this work, viz.; that during osmotic hemolysis no solutes are lost from the cell, must be taken into account, even though for moderate degrees of hypotonicity (short of hemolysis) no such loss seems to occur (PONDER and ROBINSON 1934). Nucleated erythrocytes are not known to be qualitatively different from mammalian erythrocytes in osmotic behavior, but such quantitative data as we have show quantitative differences. HAMBURGER (1898) studied the erythrocytes and sperm of a frog and the erythrocytes of the hen. The latter responded to changes in osmotic pressure like those of the horse, giving, calculated non-solvent volumes of 52.14 to 57.7%. Both erythrocytes and sperm of the frog displayed volume changes corresponding to non-solvent volumes of about 75%: for the erythrocytes HAMBURGER gives values lying between 72.0 and 76.4%. Like this author's deductions concerning mammalian erythrocytes, these figures probably need revision, but they serve to show that the high non-solvent volume found for the frog erythrocyte is not necessarily peculiar to the nucleated condition.

In this connection, however, it is interesting to note the high amino-nitrogen content of nucleated as opposed to non-nucleated erythrocytes. This has been shown by COLLIP (1920b) among others, and attributed by him to the presence of amino acids in nuclei to the almost complete exclusion of such other osmotically active substances as inorganic electrolytes. Since the amino acids are weak electrolytes, which by changes in their dissociation might counteract the effects of dilution we might expect them to cause the nuclei to take in or give up more water to produce a given change of osmotic pressure than would systems owing their osmotic pressure to strong electrolytes. This would serve to exaggerate the changes in volume due to changes in external osmotic pressure, and hence to reduce the apparent non-solvent volume. No such effect is demonstrated by HAMBURGER's experiments, nor are there any experiments to show whether the weakly basic and acidic dissociations of the presumptive amino acids have any effect on the volume changes of nucleated erythrocytes to changes in external pH. (See BECK and SHAPIRO 1936).

That the non-solvent volume of nucleated erythrocytes does not in general greatly exceed that of mammalian erythrocytes

is made more probable by COLLIP's findings (COLLIP 1920a) as to the changes in freezing point induced by twofold dilution of erythrocyte sediments. Here non-solvent material would tend to magnify the change in freezing point. COLLIP finds the non-solvent space for eight species of mammals, thus calculated, to range from 21.2 to 32.8, while that for fowl, pigeon, and turtle ranged from 27.0 to 35.7. The values given for mammalian erythrocytes are so low as to arouse doubt, and for this reason it is doubtful whether the slightly higher range for the nucleated cells represents any real difference.

One other characteristic described so far only in the case of nucleated erythrocytes may be mentioned, namely; the contraction of the hemoglobin into masses, leaving a margin of colorless stroma. This was noticed by HAMBURGER (1887) when he placed erythrocytes of hen, frog, or fish (*Tinea* sp.) in both hyper or hypotonic solutions, but was absent over an intermediate range of approximately isotonic concentrations. NaCl, KNO₃, and sucrose solutions were used.

This may have been the same phenomenon as that subsequently shown by STEWART (1902) to occur in nucleated erythrocytes, especially those of *Necturus*. In this case hemoglobin was shown to crystallize within the cells, the size and perfection of the crystals varying with the conditions. It is quite possible that these nucleated cells allow salts to leave the cell in hypotonic solutions, and thus leave hemoglobin insoluble. Either these cells allow an unusually large loss of salts, or more probably the *Necturus* hemoglobin is specially insoluble in the salt-poor cell fluid.

There seems to be no reason to consider these as osmotic phenomena, nor to assume any marked difference in osmotic behavior between mammalian and nucleated erythrocytes. Both may be considered to act as perfect osmometers, at least to a first approximation, so long as they remain normal. Even when they are known to be abnormal it has not been shown that the transfer of water into or out of the cell is due to any but osmotic forces. In evaluating the permeability to water of erythrocytes of all kinds, we shall include as a driving force only the activity gradient of the water.

The absolute permeability of erythrocytes to water. The only real quantitative approach to a determination of the

absolute permeability of erythrocytes to water has been that of JACOBS (1927, 1932, et seq.), who has measured the times necessary for a selected degree of hemolysis of erythrocytes of many vertebrates to occur in pure water and various solutions. He had previously devised special methods for accurate measurement of rapid hemolysis (JACOBS 1930). The calculation of the permeability constants involves knowledge of the volume and surface area of the erythrocytes, both initially and at the selected hemolytic endpoint. The initial values are more or less well known for the erythrocytes of many mammals (PONDER 1934), and as a first approximation it seems permissible to assume their surface areas to remain constant over the range of volumes utilized. This is because when their volume starts to increase the biconcave shape first changes to a spherical one without increase of surface, and only thereafter does swelling necessitate an increase in area. Their final volumes, however, may or may not correspond to those calculated, since they cannot be measured directly, and when measured indirectly they may depend upon other factors besides osmotic pressure. JACOBS (1932) assumes that erythrocytes in distilled water have the same volumes at 75% hemolysis that they would have in an ideal hypotonic solution just dilute enough to hemolyse 75% of them, and in which their volumes (after allowance for non-solvent volume) were inversely proportional to the osmotic pressure of the suspension media, i. e. $\Pi (V-b) = \pi_0 (V_0-b)$. It is not certain that this assumption is correct, and furthermore PONDER and ROBINSON (1934) have shown that even for incipient hemolysis rabbit erythrocytes are "imperfect osmometers", acting as though only a part of their contained water was "free". This apparent fraction ("R" in PONDER's notation) varies from about 0.45—0.50 in hypotonic NaCl solutions to 0.75 in dilute plasma, or even to about 1.0 when the dilution is not great enough to produce any hemolysis. PONDER and ROBINSON attribute the small values of this term, R, to a "leakage factor" allowing loss of osmotically active substances from the erythrocytes, and believe that it occurs also during the process of rapid and complete hemolysis such as occurs in distilled water. JACOBS (1932) on the other hand, believes that the low values found in hypotonic solutions of non-electrolytes are the effects of disturbed ionic equilibria, and assumes that neither these effects nor leakage occur during rapid hemolysis

in distilled water or dilute solutions of non-penetrating non-electrolytes.

The cause of these anomalous volume changes is not so important in the present connection as their magnitude; it is absolutely essential to know how much the cells swell, since this measures the water intake. Furthermore, leakage of osmotically active substances out of the erythrocyte would lessen the (osmotic) driving force for water. Since we cannot measure the volume changes during an actual experiment, and since even the best indirectly determined volume changes (PONDER and ROBINSON 1934) are subject to the reservation that the lecithin used to make the cells spherical may alter their volume, it will be seen that the calculated permeability constants cannot be regarded as exact. This is recognized by JACOBS.

The permeability constant for water calculated by JACOBS (1927) neglected non-solvent volume, and has since been recalculated with this in mind (JACOBS 1932). The equations and values given in the earlier paper may, therefore, be neglected here. JACOBS (1932) calculates the permeability constant by the following equations (our notation):

$$\frac{d(V-b)}{dt} = PA (C_1 - C_e) \quad \text{Equation (1)}$$

where $V-b = (V_0 - b) \frac{C_{01}}{C_1}$, C_{01} is the initial C_1 and both C_{01} and C_1 refer to total osmolar concentrations in the water of the erythrocyte. If swelling occurs in a large volume of distilled water, so that $C_e = 0$, and A , the area of cell surface, is assumed to be constant, equation (1) may be integrated to the form:

$$P = \frac{C_0(V_0-b)}{2At} \left(\frac{1}{C_1^2} - \frac{1}{C_{01}^2} \right) \quad (2)$$

from which P may be calculated. The time required for 75% hemolysis of erythrocytes from defibrinated ox blood added to distilled water was 1.4 seconds, from which $P = 0.036 \mu^3 \cdot \mu^{-2} \cdot \text{sec}^{-1} \text{ atm}^{-1} \cong 1.59 \times 10^{-9} \text{ GM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} (\text{GM l}^{-1})^{-1}$. For human erythrocytes the corresponding value of P was found to be 2.56×10^{-9} . In a subsequent paper JACOBS (1934) reports a very similar value for the permeability of ox erythrocytes: 1.82×10^{-9}

Judging from the work of PONDER and ROBINSON (1934) more normal values would be expected if hemolysis could be studied in dilute plasma or serum, since in them the cells are more nearly perfect osmometers, as shown by higher values of PONDER's R . Moreover, JACOBS (1932) has shown that his combined k' , which in our notation equals PA/V_0 , increases with decreasing hypotonicity as shown by Figure 5. Since A (if it is constant) and V_0 are initial values they should not depend upon the con-

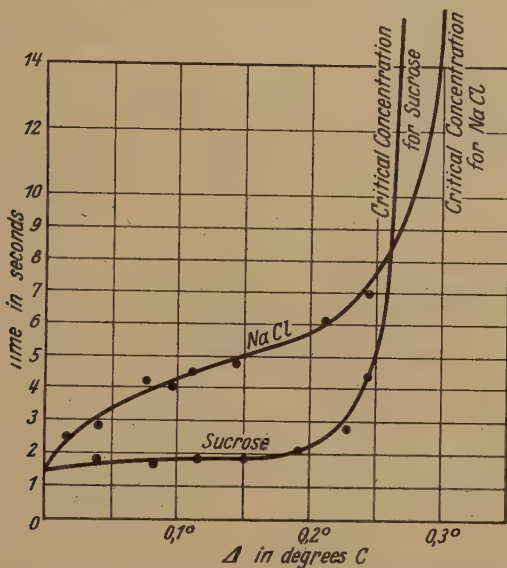


Figure 5. Rate of hemolysis of ox erythrocytes in solutions of sucrose and of NaCl.

centration of the hemolysing solution, and either the selected degree of hemolysis must correspond to different amounts of swelling in the different solutions or else erythrocytes are more permeable to water the higher the concentration of sucrose or other non-electrolyte.

JACOBS and PARPART (1932) show that in very dilute solutions of strong electrolytes (< 0.07 N) hemolysis is much slower than in sucrose solutions of comparable osmotic pressure. In the latter hemolysis proceeds at a rate not very different from that predictable on an osmotic basis alone, taking hemolysis in dis-

tilled water as the basis of comparison. In other words the calculated values of JACOBS' k' increase slowly with increasing sucrose concentration, while they are abruptly decreased by the addition of small traces of salts to distilled water; with increasing salt concentration the k' values fall off still more. The data from a single experiment with NaCl will illustrate this point (Table V). Since NaCl is responsible for most of the osmotic pressure of plasma these experiments raise a serious question

Table V. Effect of the concentration of NaCl solutions on the time required for 75 per cent hemolysis of ox blood at 20° C. One part of blood to approximately 500 parts of solution. Each time is the average of four determinations¹⁾

Concentration	Δ	Experiment 1 R = 1.63		Experiment 2 R = 1.79		Experiment 3 R = 1.69	
		Time seconds	k'	Time seconds	k'	Time seconds	k'
0.00	0.000	1.35	1.06	1.42	1.34	1.30	1.23
0.005	0.018	2.48	0.62	3.00	0.64	2.28	0.73
0.01	0.036	2.72	0.58	3.75	0.55	2.68	0.64
0.02	0.072	3.60	0.48	4.82	0.48	3.78	0.51
0.03	0.107	4.70	0.41	5.65	0.46	4.68	0.46
0.04	0.142	5.12	0.42	6.15	0.48	5.05	0.48
0.05	0.177	5.65	0.43	6.88	0.49	5.60	0.50
0.06	0.211	6.40	0.44	7.85	0.52	6.68	0.48
0.07	0.246	7.80	0.44	10.62	0.47	7.48	0.53
0.08	0.280	11.02	0.39	35.90	0.20	10.78	0.48
0.09	0.314	30.22	0.20	—	—	130.	0.06

as to whether values of k' observed in distilled water are normal. The absence of electrolytes may lead to abrupt precipitation of proteins in the plasma membrane. The plasma membrane is formed by proteins in conjunction with lipins and fatty substances (SCHMITT, BEAR and PONDER, 1938) (SCHMITT and WAUGH, 1939) and any such change in the proteins may well be reflected in the permeability constants. Values of P calculated from JACOBS' experiments with intermediate concentrations of NaCl (0.02—0.07 M) are in the neighborhood of 0.6×10^{-9} .

¹⁾ Table I by M. H. JACOBS and A. K. PARPART. The Biological Bulletin, 63: 228, Oct. 1932.

PONDER (1934) has given values of permeability for sheep erythrocytes in 0.096—0.126 M NaCl varying from 0.69 to 5.83×10^{-7} .

It is evident that permeability to water ought to be measured under more nearly normal conditions than have yet been used. It should for example be possible to measure the time required for some relatively small degree of hemolysis to occur in diluted whole blood (e. g. of the horse) without the use of anticoagulants or defibrination.

The values of P so far obtained, viz. 1.59— and 2.56×10^{-9} are, therefore, provisional. Refinements of method will probably modify them materially, but the true values are unlikely to be much less than 10^{-9} or more than 10^{-8} for most mammalian erythrocytes.

Variation in the permeability to water among erythrocytes of different species. The times required for the hemolysis in distilled water of the erythrocytes of different species of vertebrates vary greatly. This is well shown by JACOBS (1931), who tested 38 species, including elasmobranch and teleost fishes, amphibians (Anura), reptiles (Chelonia), birds, and mammals (rodents, ungulates, carnivores and man). Outstanding features are the very slow hemolysis for elasmobranchs, frogs, and among birds the domestic fowl; pigeons, ducks, most teleost fishes and all mammals gave rapid hemolysis; mouse and sheep erythrocytes hemolysed most quickly. Although these differences may be due at least in part to the difference in permeability, other properties of the erythrocytes are almost certainly important. Thus PONDER (1935) found that the differences in "fragility" of the red cells of various mammals can be accounted for almost entirely on differences in their critical hemolytic volumes, that is, in the amount of water which they can take in without losing their hemoglobin. This in turn is correlated with the initial volumes of the erythrocytes, and is affected by the surrounding medium. For hemolysis in water it may be noted that the large erythrocytes of the elasmobranchs (ca. $27 \times 19\mu$) and frogs (ca. $24 \times 16\mu$) hemolysed slowly in JACOBS' experiments, while the small ones of pigeons and ducks (ca. $12 \times 8.5\mu$) and mammals (diameter ca. 5 to 8μ) hemolysed rapidly. The results with domestic fowl and land turtles appear anomalous. The fact that erythrocytes of the fowl require extreme

dilution of NaCl solutions for hemolysis (CORDIER 1930) suggests that they may have a very large critical hemolytic volume. Among mammals there appears to be a correlation between speed of hemolysis, fragility and the volume/surface ratio. The correlation between the last two has been discussed by PONDER (1935)¹. JACOBS, GLASSMAN and PARPART (1938) have examined the relative speeds of hemolysis of the erythrocytes of two closely related species, the rat and the mouse. Isosmotic hypotonic solutions of such substances as glycerol and thiourea, glycerol and monoacetin, or ammonium chloride and benzoate were used, and the relative speeds of hemolysis for the two species were either differentially changed or even changed in inverse senses. Evidently further research is needed on the volume/surface ratios, critical hemolytic volumes and permeability to water of erythrocytes from a wide variety of vertebrates.

The relative permeability of erythrocytes to "heavy" water (deuterium oxide). Since deuterium oxide (D_2O) molecules are much like H_2O molecules in structure and have nearly the same molecular volume, it might be thought that equal concentration gradients would produce equal transfer of the two kinds of molecules through a given plasma membrane, but this seems to be untrue. (PARPART, 1935, and BROOKS, 1935).

BROOKS, S. C. (1935) measured the rate of hemolysis of sheep erythrocytes in hypotonic NaCl solutions containing D_2O and H_2O or H_2O alone, and found that D_2O penetrated less rapidly than H_2O . He considers the differences as correlated with fugacity differences in the two substances. In another set of experiments with *Nitella*, BROOKS, S. C. (1937) found that D_2O acts as a plasmolytic agent. The free-diffusion experiments of ORR and THOMSON (1934) and ORR and BUTLER (1935) on heavy water may offer some suggestions as to explanations.

ANDERSON and PARPART²) have calculated that the difference in rate of hemolysis of erythrocytes equilibrated with D_2O -NaCl solutions and transferred to D_2O -hypotonic NaCl so-

¹) Data on the dimensions of the red cells, derived in many cases from the classical paper of Gulliver (Proc. Zool. Soc. Lond., 1875, 474—95) may be found in PONDER's "The erythrocyte and the action of simple haemolysins" (Edinburgh and London, 1924).

²) Personal communication.

lutions, as compared with erythrocytes placed in H_2O -NaCl solutions and then transferred to hypotonic NaCl solutions, is 40%; whereas the vapor pressure gradient in these two cases is only 13%. This indicates that vapor pressure or fugacity is not the only factor which influences the rate of penetration of D_2O . PARPART (1937) found that hemolysis of erythrocytes (ox, rat and sheep) was less rapid in D_2O - H_2O mixtures than in H_2O alone, when the concentration gradient of water (total D_2O and H_2O) was the same, and suggests that this is due to the lower mobility and higher viscosity of D_2O as compared with that of H_2O .

BROOKS points out, however, that the vapor pressure of D_2O is so much less than that of H_2O that the total vapor pressure of a 40—60 mixture of D_2O and H_2O is about that of a 7.8% solution of NaCl in H_2O . Since fugacity is nearly proportional to vapor pressure, the 40% D_2O mixture is actually *strongly hyper-tonic* to erythrocytes which have been in serum or H_2O salt solution, and would initially withdraw water from them. This alone might account for much slower hemolysis on dilution of H_2O solutions with D_2O .

If the erythrocytes have previously been suspended for some time in an "isotonic" D_2O -NaCl solution this effect is avoided. PARPART did such an experiment with solutions 99.5% of whose water was D_2O . If the critical hemolytic and initial volumes were the same as in pure H_2O solutions, then the rate of D_2O penetration would be 0.73 times that of H_2O . Since the fugacity of D_2O at 20° is 0.87 times that of H_2O it will be seen that only part of the difference found by PARPART can really be attributed to differences in permeability, the remaining difference being about 11.5%. Difference in driving forces is therefore very important, even in the case of such similar substances as D_2O and H_2O .

Even the remaining difference, 11.5% may well be due to differences in initial or critical hemolytic volumes of the erythrocytes. One must consider the possibility that the low activity of D_2O results in changed water equilibria of gels forming part of the plasma membrane, or part of the stroma. Hydration of stroma components though probably unimportant, will also be changed.

Heavy water (D_2O) has been specially used in many biological studies within the last few years, but only a few of these

are directly concerned with permeability. Toxicity studies on white mice such as those of BARBOUR and TRACE (1936) attribute their results to high viscosity of D_2O as a factor which impedes glomerular filtration. VON HEVESY, HÖFER and KROGH (1935) measured the permeability of the skin of frogs by noting the number of days which one mole of heavy water required to pass through 1 sq. cm. at a constant difference of 1 mole concentration. The permeability was found to be the same in both directions, and no effect of ions was observed. The rate of permeability of D_2O at 21° was found to be 100 to 170 days as compared with 22 to 37 days for H_2O .

VON HEVESY and HÖFER (1934) found that there is an exchange between the water of the body and the heavy water of the medium in which fish have been placed. McDougall, Verzar, Erlenmeyer and Gaertner (1934) have also demonstrated that heavy water distributes itself throughout the entire body of the rat within one hour.

Lucké and Harvey (1935) showed that the rate of penetration of D_2O into unfertilized eggs of *Arbacia punctulata* is the same as that of H_2O but that the D_2O inhibits egg development.

Experimental modification of the permeability of erythrocytes to water. It has already been pointed out that the rapidity of hypotonic hemolysis depends not only on the degree of hypotonicity but also upon the nature and concentration of the non-electrolytes and salts present (Jacobs 1922, Jacobs and Parpart 1932b). It has further been pointed out this may or may not be due to differences in permeability. This point may now be considered in greater detail.

Non-electrolytes, such as sugars to which the erythrocytes are relatively impermeable, slightly accelerate hemolysis¹.

The following mechanisms must be considered. (1) the critical hemolytic volume is decreased. Ponder and Robinson (1934a) have found that under some conditions the erythrocytes from a single sample of blood assume different volumes (as judged by measurement after they are made spherical by the addition of

¹) The effects of penetrating non-electrolytes will involve an acceleration of hemolysis due to change in driving force (osmotic pressure difference) whose results in special cases may be calculated from the relative permeability of the cell to water and solute. See Chapter IX.

lecithin) in different solutions, each of which will just cause barely perceptible hypotonic hemolysis. Only NaCl solution, serum and oxalated plasma were studied. We do not know the critical hemolytic volumes of erythrocytes in solutions of sucrose and similar non-penetrating non-electrolytes. Altered critical hemolytic volume may, therefore, occur and simulate altered permeability.

(2) There may be an exchange of anions by which OH^- from the suspending medium enters and HCO_3^- leaves the erythrocyte. This ionic readjustment seems inevitable if the erythrocyte is as permeable to these ions as the changes during transformation of arterial to venous blood and the reverse seem to show (Chapter XII). Furthermore it is in the right direction to account qualitatively for the observations. JACOBS (1932) points out that the consequences of such an exchange would be increasing intracellular pH and transfer of base from HCO_3^- to hemoglobin. "If the compound of base with hemoglobin be represented as $B_n Hb$ the osmotic pressure of this compound when completely dissociated would be to that of the same amount of base combined with carbonic acid" (as bicarbonate) "as $(n + 1) : 2n$ ". The exchange of anions would therefore, since $n > 1$, reduce the osmotic pressure of the erythrocyte contents and thus reduce the concentration just able to produce finally a given degree of hemolysis. This process seems to occur in less than five seconds. But if hemolysis occurs within less than two seconds, as it does in distilled water or very dilute non-electrolytes, there is not enough time for even this rapid readjustment to occur. The way in which this might operate through JACOBS' equations to give the observed apparent increase in P with increasing concentration cannot be stated briefly and the reader is referred to the original paper. (JACOBS 1932). But it has not been shown that it accounts quantitatively for the observed effect.

(3) Electrolytes may be lost from the erythrocyte within the first few seconds of swelling. Some such effect is suggested by YEAGER's experiments (1931) on resistance to hypotonic hemolysis in solutions of various sugars. But these experiments do not show how quickly the process occurs, if at all. In dilute NaCl some osmotic readjustment occurs within a few seconds (about 15) as PONDER and ROBINSON (1934) have shown. They found that the final volume of rabbit erythrocytes in NaCl solution

diluted to 0.68% was assumed within this time, and that it was less than would be calculated on an unmodified osmotic basis, i. e., in their experiments $\Pi (V - b) = \pi_0 (V_0 - b)$. Since subsequent analysis showed that K had left the erythrocytes (which contain no Na) and appeared in the solution (which originally contained no K) in roughly the right amounts to account for the observed discrepancy, the authors conclude that the initial rapid osmotic readjustment was due to loss of KCl. It seems conceivable that the initial readjustment was of the type suggested by JACOBS (1932) and discussed under (2) above, and that the loss of K was due to a subsequent exchange of K for Na. The experiments of BROOKS (1939) show the reality of rapid ion exchanges for *Nitella* within one minute for K. The apparently negative finding of COHN and COHN (1939) using Na and dog erythrocytes may be attributable to the negligible reactivity of hemoglobin at the pH of the blood stream, and the relatively low ratio of the stroma proteins to the cell's hemoglobin (See Chapter XI). Against this interpretation may be urged the fact that the erythrocytes had been washed twice in isotonic NaCl solution before V_0 was determined, and that the anion shift should have occurred before this. It therefore remains debatable whether loss of electrolytes during the initial stages of swelling occurs in dilute solutions of non-electrolytes, or even in dilute salt solutions. More conclusive evidence is needed.

(4) Finally, to whatever extent factors (1), (2) and (3) fail to account quantitatively for the observed apparent change in P for water with change in non-electrolyte concentration we may assume that the true permeability actually does not change. It is evident that we are still a long way from knowing that any such change occurs.

Further references bearing upon this subject are cited by JACOBS (1932) and discussed by JACOBS and PARPART (1933) and JACOBS (1935).

Narcotics include a number of substances belonging to such groups as the alcohols, ethers, urethanes, chloroform, etc. In the higher concentrations they may be considered as members of a larger group of hemolysins which comprise also such substances as fatty acids, soaps, bile acids and their salts, saponins, bacterial toxins and venoms. The increased permeability to practically all substances which characterizes the effects of these

hemolysins may be classed as abnormal, and of interest only insofar as it sheds light on the composition of the plasma membrane.

Narcotics in low concentrations have been thought to decrease the permeability in general, and in particular to decrease permeability to water. (TRAUBE, 1908; ARRHENIUS and BUBA-NOVIĆ, 1913; YOSHITOMI, 1920; and JARISCH, 1921). All of these authors confuse the final "equilibrium" degree of hemolysis with the rate of its attainment, which latter alone defines permeability. JACOBS and PARPART (1932a) give an excellent critique of the error thus made by these authors. In addition they show that ethyl-, n-butyl-, i-amyl- and phenylurethanes added to distilled water in low concentrations retard hemolysis. The retardation of hemolysis seems greater than any possible effect on the amount of water which is taken in by an erythrocyte in coming to its final volume in the urethane solutions. But no measurements of final volume are possible in those solutions in which differences in rate of hemolysis are measurable. It may well be that the change in absolute rate of entry of water is merely proportional to an (undeterminable) change in driving force, and that no change in permeability actually occurs. Other explanations of the retardation of hemolysis by urethanes are also discussed by JACOBS and PARPART, and a tentative hypothesis advanced to account for the change in permeability, which the authors suggest may perhaps be in part responsible for retardation of hemolysis. This hypothesis will be mentioned again in connection with speculations as to the nature of the plasma membrane.

Strong electrolytes appear to have a definite effect upon permeability, but here again it is impossible to be sure that changes in critical hemolytic volume or in driving force are not responsible. Obviously the extensive literature upon the fragility of erythrocytes in solutions of different salts, as determined some hours after the suspensions have been made up, tell us nothing about permeability to water. In the case of water, substantial equilibrium is obtained within a few seconds or minutes at most, and any water exchange thereafter depends upon and goes on at a rate determined by some disturbance of the equilibrium. The same qualification applies to most if not all experiments in which salts have been used in conjunction with other hemolysins, which in any event are primarily studies of the rate

of action of the hemolysin. These also tell us nothing about permeability to water.

JACOBS and PARPART (1932b), however, have shown that several salts markedly delay rapid hypotonic hemolysis. The salts used included NaCl, Na_2SO_4 , MgCl_2 , MgSO_4 , CaCl_2 and Al salt. The retardation of hemolysis may be seen from Fig. 6 taken from the paper by JACOBS and PARPART. The lowest curve, marked S, is that for sucrose alone. This shows that the effect depends primarily upon the valence of the cation, the nature of

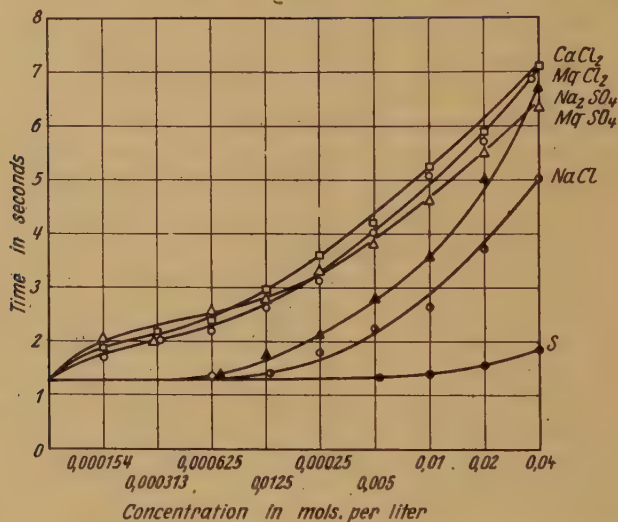


Figure 6. Rate of hemolysis of ox erythrocytes in solutions of various salts.

the anion being of secondary importance. The Al salt showed a retarding effect in concentrations as low as 10^{-5} M.

The similarity between these curves and those obtained by LOEB (1919, 1920, 1922) for the rate of osmosis of water through gelatin coated collodion membranes into salt solutions suggests that the two sets of phenomena have the same basis, namely, electroosmosis. The changed ionic environment of the erythrocyte may lead to considerable potential gradients across its plasma membrane, and these in turn might, as driving forces, perceptibly affect the rate of entry of water into the erythrocyte.

This explanation is tentatively offered by JACOBS and PARPART after discussion of other possible interpretations. Insofar as these experiments concern true permeability to water we may conclude that no effect of ions has been shown to exist. It should be remembered that any ion exchange between the plasma membrane and the surrounding fluid may affect the solubility of the proteins, and thereby affect the permeability.

The effects of certain colloids on the resistance of erythrocytes to hypotonic hemolysis have been studied by SPRANGER (1930)¹, with special reference to the opposed effects of hydrophile colloids (lecithin, serum albumen, sodium oleate) and hydrophobe colloids (cholesterol and its esters, triolein). Erythrocytes treated with an isotonic solution containing, e. g., lecithin swell more on subsequent transfer to hypotonic NaCl solutions, and shrink more in hypertonic solutions. Hydrophobe colloids, such as cholesterol have the opposite effects. Corresponding to these disturbances in the equilibrium swelling SPRANGER found that lecithin treated cells were hemolysed in more concentrated NaCl solutions, i. e., were more "fragile", while cholesterol treated cells were more resistant. These effects are evidently due to a disturbance in water equilibrium, the mechanism of which is yet obscure. It does not necessarily involve any change in permeability, although SPRANGER attributes the effects solely to such a change. If permeability alone had been affected, the equilibrium volumes of the cells would have been unaffected, but the process of swelling (hemolysis in sufficiently dilute solutions) should have been accelerated. This SPRANGER has not demonstrated, and we cannot, therefore, decide whether substances such as he used change permeability or not. Earlier work on non-living systems (SPRANGER 1929) suggests that they possibly do. Another possible factor in SPRANGER's experiments is the Donnan equilibrium effect of colloidal ions (albumen, lecithin) on the osmotic balance between cells and surrounding fluid. Such an effect has been observed in the case of sea urchin eggs by LOEB (1908) but SPRANGER's experiments are too complex to permit of a satisfactory explanation in terms of permeability to water

¹) See also BRINKMAN, R., and E. VAN DAM, *Biochem. Z.* 108: 35—51 (1920) and 108: 52—60 (1920). For a discussion of the relation of lecithin and cholesterol to permeability to other substances see later chapters.

Other factors which presumably affect the permeability of the erythrocyte to water include pH, temperature and such physical factors as radiation and pressure. In no case can we assign numerical values to the alterations in permeability, if any, produced by these agents. Since acids, freezing, heating, ultra-violet light, etc., may cause hemolysis they presumably affect permeability.

In the case of two of these factors, viz.: pH and temperature, we have a quantitative study by JACOBS and PARPART (1931) of their effects on final amounts of hemolysis, which in turn are assumed to depend upon differences in internal osmotic pressure produced by these agents. Oxygen also enters as a factor since the internal osmotic pressure depends upon the amount of base bound by hemoglobin and oxyhemoglobin. These are acids of different strength, and the dissociation of each is a function of pH and temperature. Significant changes in the final amount of hemolysis may be caused by differences as little as 0.01 pH unit, or 0.5° C. The effects of temperature on the penetration of water and other substances have been studied recently in extenso by JACOBS, GLASSMAN and PARPART (1938). Using different species of erythrocytes, they found that the mean calculated Q_{10} varied from 1.24 to 1.40 from 0° C to 40° C. The effects of external pH on the volume of erythrocytes, based upon EGE's estimates of non-solvent volume have been studied by WARBURG (See Table II) in exceedingly careful experiments, and have been found to agree well with observation. (See also Table 10 from VAN SLYKE, WU and MACLEAN 1923). These conclusions invalidate all quantitative findings in those experiments in which pH and temperature have not been closely controlled. It is disappointing to note that tests are still being made in unbuffered salt solutions at "room temperature"!

Other methods which have been used for the determination of bound water (which might be considered equivalent to the non-solvent volume) in erythrocytes are beyond the scope of this monograph. Among those used may be mentioned those of DIAZ, BIELSCHOWSKY and MINON (1935), SLAWINSKI (1933, 1934), and PARPART and SHULL (1935). As opposed to these may be mentioned the paper of MACLEOD and PONDER (1936), who believe that all the cell water is solvent and attribute extraneous results to errors in the methods used.

CHAPTER IV

OSMOTIC EQUILIBRIUM IN THE EGGS OF MARINE INVERTEBRATES

The eggs of marine invertebrates are peculiarly favorable for the study of osmotic equilibria. Although erythrocytes are better in lacking nuclei and possibly in having a less complex cytoplasm, their volume changes are difficult to follow with certain freedom from error. Many eggs on the other hand are spherical, and retain their sphericity over a considerable range of osmotic pressures; hence measurement of one diameter of one of these spherical cells suffices to establish its absolute volume. Our knowledge of the osmotically produced changes in volume of these eggs is, therefore, relatively exact, but the analysis of these changes is more difficult than in the case of the erythrocytes.

Three principal methods have been used to measure the diameters of these eggs: direct visual measurement with an ocular micrometer, used by most workers; photomicrography followed by direct measurement of the photographic image (LEITCH 1936); and an adaptation of the diffractometric method developed by PONDER and SASLOW (1931) for erythrocytes, which has been used by LUCKÉ, LARRABEE and HARTLINE (1935) for eggs of *Arbacia punctulata*. Essential agreement obtains between these methods.

LILLIE (1916) was apparently the first to make quantitative studies of the osmotic volume changes of the eggs of marine invertebrates. HARVEY (1910) had already called attention to these changes, but studied rates of swelling only, and not equilibria. LILLIE found that in a mixture of 40 parts sea water with 60 parts tap water the average volume of eggs of the sea urchin *Arbacia punctulata* was $40.4 \times 10^{-8} \text{ cm}^3$, as compared with 20.6×10^{-8} in sea water. If the relation: $\Pi \text{ ext} \cdot V = \text{constant}$ were

valid the volume in dilute sea water should have been $51.5 \times 10^{-8} \text{ cm}^3$. The swelling, like that of erythrocytes, was, therefore, less than inversely proportional to the osmotic pressure. LILLIE attributed this behavior to the influence of what we have termed non-solvent material.

These observations were subsequently extended by LILLIE (1918) to other osmotic pressures, including hypertonic solutions, with no marked difference in result so long as crenation did not occur. This result has been confirmed for *Arbacia* by MCCUTCHEON and LUCKÉ (1926), and corresponds qualitatively to FAURÉ-FREMIET's observations on eggs of the annelid *Sabellaria alveolata*. (FAURÉ-FREMIET 1923, 1924). In this case the eggs were placed in hypo- and hypertonic solutions of sucrose in which they swelled or shrank much less than in proportion to the osmotic pressure changes. FAURÉ-FREMIET explained this behavior, as HAMBURGER had that of erythrocytes (See previous section), by assuming a certain part of the egg to be osmotically inert. If this non-solvent volume was considered to be equal to the volume of dry matter in the egg as determined by chemical analysis (FAURÉ-FREMIET 1921) namely, 30 per cent, then the observed volumes corresponded to the first approximation to those calculated, i. e., $\Pi (V - b) = \Pi_0 V_0 - b$.

Here we should mention an experimental difficulty often underemphasized. No satisfactory method of distinguishing analytically between the intracellular water and the unknown amount of intercellular water included in any mass of these or similar eggs has yet been described. FAURÉ-FREMIET centrifuged the eggs and dried the sedimented mass with filter paper or else he filtered the mass with suction. He regarded the intercellular water as being incompletely removed, and the analytically determined water content as too high. But there is also a real danger that water may be squeezed or absorbed out of the cells by these methods. The liability of these and similar marine eggs to cytolysis increases the uncertainties. EPHRUSSI and RAPKINE (1928) have tried to avoid this source of error by centrifuging the spherical eggs so lightly that they just made contact without distortion. The amount of intercellular sea water in such a mass could then be estimated from the known packing correction for close packed spheres, and the proper corrections applied to analyses of such sediments. However, some eggs deform and break up even under

1 \times gravity, so that this method though promising is difficult to evaluate as to accuracy. Efforts have been made to estimate the amount of intercellular water by adding to the original suspension known amounts of dyes, hemoglobin or other reference substances, and after centrifugation determining how much was recovered in the decantate. But these methods have not been successful for various reasons. (LEITCH, J. L. Personal communication.) It must not be surprising, therefore, if the analytically determined dry weights do not prove to be exactly identical with the non-solvent volumes deduced from osmotic experiments.

Good agreement between osmotically and analytically determined non-solvent volumes is, as a matter of fact, only occasionally found. Table VI gives data for six echinoderms and two worms (*Sabellaria* and *Urechis*). Only in LEITCH's experiments (1934) do the data on a given line in the table refer to eggs collected at the same times. In all other cases the data were obtained at different times, and often by different workers, or even at widely separated stations. Table VII includes data on non-solvent volumes of additional forms for which no analytical data have been published.

Surveying Table VII we find good agreement in the case of *Sabellaria*, and for one batch each of *Strongylocentrotus franciscanus* and *S. purpuratus*; fair agreement in the case of *Paracentrotus* as calculated by BIALASZEWICZ, and occasional rough agreement for *Arbacia punctulata*. Disagreement is of two types: (1) osmotically determined non-solvent volume unduly high (*Arbacia pustulosa*, *Paracentrotus lividus* (EPHRUSSI and NEUKOMM) *Strongylocentrotus* spp. and *Urechis*); (2) osmotically determined non-solvent volume unduly low (*Arbacia punctulata* (many values), *Pisaster ochraceus*). In the first case the observed osmotic volume change is unduly small; in the second, unduly high. The possible explanations of these disagreements are many. Some of them have been considered by LUCKÉ and McCUTCHEON (1932), LEITCH (1934) and LEITCH (1936). Not all of them can be considered here.

Analysis may be in error because of the difficulty referred to above, of removing from the sample all of the intercellular fluid and none of the water normally present in the eggs. This may be avoided in theory at least by LEITCH's method (1934)

Table VI. Comparison of non-solvent volumes deduced from analyses with those calculated from osmotic volume changes for the eggs of a series of marine invertebrates

Species	Non-solvent volumes in % of original volume			
	From analyses		From volume changes	
	Values	Auth.	Values	Auth.
<i>Arbacia punctulata</i>	18.1	11	7—20	10
„ <i>pustulosa</i>	17.8	1	36.4	2
<i>Paracentrotus lividus</i>	20.7	1	25.1	2
„ „	22.7	4	46.0	9 (calc.
„ „	22.6	12	—	from 3)
<i>Strongylocentrotus</i>	18.2	8	17.9	8
<i>franciscanus</i>	17.9	8	28.4	8
<i>S. purpuratus</i>	24.2	8	24.5	8
	15.5	8	24.1	8
<i>Pisaster ochraceus</i>	19.9	8	0.0	8
„ „	22.4	8	—	—
<i>Sabellaria alveolata</i>	30.0	6	30.0	5.7
<i>Urechis caupo</i>	17.1	8	48.3	8
„ „	12.7	8	47.9	8

Key to References:

1. BIALASZEWICZ (1929).
2. BIALASZEWICZ (1932).
3. EPHRUSSI and NEUKOMM (1927).
4. EPHRUSSI and RAPKINE (1928).
5. FAURÉ-FREMIET (1921).
6. FAURÉ-FREMIET (1923).
7. FAURÉ-FREMIET (1924).
8. LEITCH (1934).
9. LUCKÉ and McCUTCHEON (1932).
10. LUCKÉ, LARRABEE and HARTLINE (1935).
11. McCLENDON (1909).
12. WETZEL (1907).

of multiplying the average volume of individual eggs by the number of eggs in the sample, and subtracting from this figure the calculated volume of the principal solids (proteins, lipoids and carbohydrates) found in the sample. But other sources of error enter into this method: the factor relating nitrogen to protein may differ from that (6.25) usually used, which is based on mammalian proteins; the separation and estimation of lipoids are generally unsatisfactory.

Factors interfering with simple osmotic volume changes are particularly troublesome. The unduly high non-solvent volumes given in Table VI are probably due to a good many different factors, among which the following may be mentioned.

(a) **Lack of sphericity** of the eggs would favor this result, and can be corrected for only if the vertical diameter as well as the horizontal diameters of the eggs are measured. But eggs of many of the species used are known to be usually very nearly spherical. This source of error is probably not very important in the species listed. For further discussion see LEITCH (1934).

Table VII. Non-solvent volumes deduced from the osmotic volume changes of the eggs of several marine invertebrates, and of portions of such eggs. Non-solvent volumes are given in % of the original volumes in sea water

Species	Part of egg	Non-solvent volume	Author
<i>Echinometra lucunter</i> . .	Whole	36.0	LEITCH (1936)
<i>Echinus microtuberculatus</i>	"	22.6	BIALASZEWICZ (1932)
<i>Psammechinus miliaris</i> .	"	34.8	BIALASZEWICZ (1932)
<i>Phallusia mammilata</i> . .	"	19.3	BIALASZEWICZ (1932)
<i>Ceratocephale osawai</i> . .	"	5—30	KAMADA and YAMOMOTO (1931)
<i>Arbacia punctulata</i> . . .	Whole	9.1	LUCKÉ (1932)
" " . . .	Centripetal half	7.4	LUCKÉ (1932)
" " . . .	Centrifugal half	16.5	LUCKÉ (1932)
<i>Asterias forbesii</i>	Cytoplasm	ca. 26.5	BECK and SHAPIRO (1936)
" "	Germinal vesicle	ca. 0.0	BECK and SHAPIRO (1936)

(b) **Resistance of the surface or outer envelopes of the eggs** to stretching when swelling occurs in hypertonic solution. This is illustrated by the eggs of *Urechis caupo*, and those of *Ceratocephale osawai*. *Urechis* eggs, whose swelling was studied by LEITCH (1934), are known to have a tough surface membrane which resists penetration of micro-needles (TAYLOR 1931). It is not surprising that these eggs were found to swell less than would be expected on the basis of their analytically determined non-solvent volumes, thus yielding a high volumetrically determined non-solvent volume. Eggs of *Ceratocephale* were examined by KAMADA and YAMAMOTO (1931). They found the non-solvent volume to increase with dilution of the surrounding medium, as shown in Table VIII, which is adapted from their figures. They calculated by the method on FRANK (1906) what surface force would account for the failure of the eggs to swell or shrink as much as the average non-solvent volumes demanded when they were put into a standard balanced salt solution diluted to $\frac{1}{4}$, $\frac{1}{5.38}$ and $\frac{1}{8}$, the second value being that regarded as isotonic. They conclude that the effects of any such surface force are too small

Table VIII. Non-aqueous space in the cytoplasm and the relative amount of free solutes within the cell.

(Diameter in cm., osmosis in atmospheric pressure in the calculation)
After KAMADA and YAMAMOTO (1931)

Conc.	x (vol. of non-aqueous phase)
"1"	$(5 \times 10^{-7} \pm 0.3 \times 10^{-7} \text{ cm}^3)$
$\frac{3}{4}$	$5 \times 10^{-7} \pm 0.2 \times 10^{-7} \text{ ,,}$
$\frac{1}{2}$	$6 \times 10^{-7} \pm 0.2 \times 10^{-7} \text{ ,,}$
$\frac{1}{4}$	$7 \times 10^{-7} \pm 0.1 \times 10^{-7} \text{ ,,}$
$\frac{1}{8}$	$11 \times 10^{-7} \pm 0.2 \times 10^{-7} \text{ ,,}$
$\frac{1}{16}$	$23 \times 10^{-7} \pm 0.3 \times 10^{-7} \text{ ,,}$
$\frac{1}{32}$	$(30 \times 10^{-7} \pm 0.3 \times 10^{-7} \text{ ,,}$

to detect. But Fig. 7 shows that the vitelline membrane, which is so inelastic that it remains unchanged as the egg cytoplasm shrinks away from it in hypertonic media, may well exert a pro-

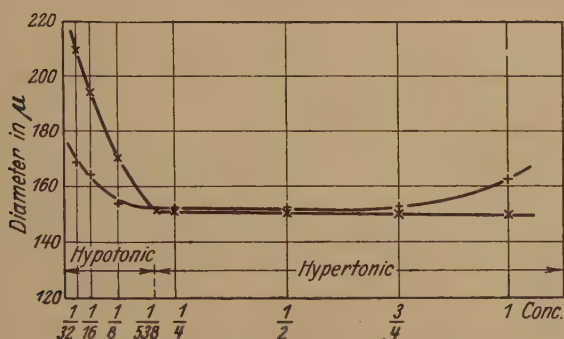


Figure 7. Vitelline membrane at various osmotic pressures of the egg of *Ceratocephale osawai*.

nounced restriction of swelling in media of concentration less than $\frac{1}{8}$. It is only in this range that the calculated non-solvent

volumes increase markedly, and this increase may, therefore, safely be attributed to the rigidity of the vitelline membrane.

As slight drift of non-solvent volume to higher values in swollen eggs might conceivably be accounted for by an increase in hydration with increase in the activity of intracellular water (Chapter II.), but in view of existing doubts about the amount of water thus bound in protoplasm, this explanation seems doubtful. It might equally well be supposed that a very slight force in the surface of the cytoplasm was responsible. Such forces have been measured for many eggs of marine invertebrates, and in general seem to be too small to be detected by existing methods of measuring osmotic volume changes. Their values appear to lie between $0.08 \text{ dynes} \cdot \text{cm}^{-1}$ for *Arbacia* eggs (COLE, 1932) and $1.1 \text{ dynes} \cdot \text{cm}^{-1}$ for *Illyanassa* (HARVEY, 1931b; see also HARVEY, 1931a). The value of about $20 \text{ dynes} \cdot \text{cm}^{-1}$ calculated by VLÈS (1926) for the eggs of *Paracentrotus lividus* is probably erroneous. (See COLE, 1932).

(c) In the case of *Paracentrotus lividus* BIALASZEWICZ (1932) obtained fair agreement between the non-solvent volumes calcu-

ated from shrinkage in hypertonic media (HERBST's artificial sea water (HERBST 1904) made up in higher concentrations) and those previously deduced from chloride and freezing point determinations made on ultrafiltrates from various dilutions of crushed eggs. (BIALASZEWICZ 1929). Total dry matter determinations by EPHRUSSI and RAPKINE (1928) and WETZEL (1907) using eggs of the same species are in even better agreement with BIALASZEWICZ' volumetric determinations.

But EPHRUSSI and NEUKOMM (1927) observed that those eggs (*Paracentrotus lividus*) in diluted sea water or in sea water made hypertonic by the addition of sucrose seemed to resist swelling or shrinkage respectively. LUCKÉ and McCUTCHEON (1932) have plotted the volumes observed by EPHRUSSI and NEUKOMM (after 45—60 minutes) against the inverse osmotic pressures, and by extrapolation to $1/\pi = 0$ deduced a non-solvent volumes of 46%. Having found similar unduly high values of non-solvent volume for *Arbacia* eggs which proved to have been injured, i. e., to have become incapable of fertilization, LUCKÉ and McCUTCHEON attribute the high value in EPHRUSSI and NEUKOMM's experiments to injury.

The nature of this injury is made more clear by the following observations. LEITCH (1934) had noticed that vacuole formation occurred in connection with the swelling of eggs of all the forms

which he had studied (See Table VI). A more detailed study (LEITCH 1936) of an additional species, *Echinometra lucunter*, showed that in this case a preliminary volume equilibrium was reached after about an hour in 60% sea water, and was maintained for perhaps 20 minutes. Then a decrease in egg volume occurred and was accompanied by the formation of abundant small vacuoles which moved toward the egg surface. At the same time the eggs ceased to be capable of developing if fertilized, and although anew and smaller "equilibrium volume" was

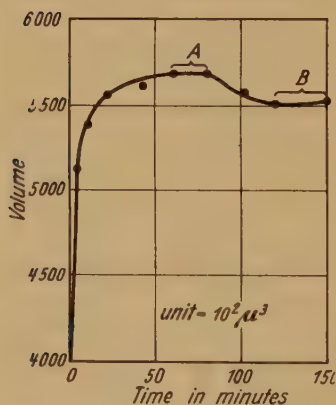


Figure 8. Volume changes at different times of *Echinometra lucunter*.

maintained for a short time cytolysis always occurred about 2 to 3 hours after the eggs were first put in the diluted sea water. Fig. 8 shows these volume changes, and shows how the relatively constant volume maintained at 90 to 120 minutes might, if earlier readings had not been taken, have led to exaggerated estimates of the non-solvent volume.

This active fluid output by vacuolization must be related in some way to the vacuole formation observed by JUST (1929) when *Nereis* eggs which had been kept for some time in hypotonic sea water were returned to normal sea water. There is also a suggestive resemblance to the active fluid output by *Spirogyra* spp. and *Vampyrella lateritia* as described by LLOYD (1926a, 1926b), although in these cases the process is a normal part of the vital activity of the cells. The composition and osmotic pressure of the excreted fluid is not known in any of these cases, and it cannot be definitely decided whether or not osmotic work is done in the process. Various possibilities are discussed by LERTCH (1936).

If the eggs in question excrete an isotonic solution, then it would seem probable that on return to normal sea water the eggs should fail to regain their initial volumes. Indeed, the capacity to regain their original volumes has been used by LUCKÉ (1932), for example, as proof of the fact that eggs were uninjured, and that their volume in diluted media was unaffected by loss of solutes. Nevertheless, it remains unproved that eggs which have recently lost fluid by vacuolization in dilute media do not take in both salts and water on return to more concentrated media. Pending such proof LUCKÉ's criterion, while plausible, needs further evidence.

(d) Finally we have to reckon with electroosmotic forces which almost certainly exist, but whose magnitude is quite unknown. Insofar as other factors, notably those given above under (a), (b) and (c), fail to account for observed discrepancies between analytically and osmotically determined non-solvent volumes, we are free to guess that electroosmotic forces are involved. But experimental support for such a guess is lacking.

Another group of species furnishes data for Table VI which indicate osmotic volume changes in excess of those predicted from analysis: i. e., the non-solvent volume calculated from volumetric data is less than that determined by analysis. In this

group are *Pisaster* and *Arbacia punctulata*. In the case of *Arbacia* the volumetric data seem particularly good in view of the care used and the extremely large numbers of eggs studied. (McCUTCHEON, LUCKÉ and HARTLINE 1931; LUCKÉ, LARRABEE and HARTLINE 1935). The non-solvent volumes calculated on this basis vary from 7 to 20% in different experiments. Since only a single analysis of these eggs is recorded (McCLENDON 1909) it is impossible to say whether or not equal variation occurs in the actual dry matter content of the eggs of different females or at different seasons. The tabulated differences in the case of *Arbacia* may well be meaningless. More analyses are needed, especially of the eggs of different individual females.

In the case of *Pisaster* however, there is good reason to surmise that there is a real basis for the tabulated discrepancy. LEITCH (1934) discusses various possible explanations, of which the most important is the possible occurrence of a type of secondary swelling which superficially resembles that observed in the case of swelling gelatin gels by NORTHROP (1927) and attributed by him to fatigue of the elastic gel structure. LEITCH (1934) observed a sudden increase in the rate of swelling of *Pisaster* eggs in 30 to 40% sea water at about the fifteenth minute. It is quite possible that this corresponds to either a breakdown of internal or membrane restraints, or to the formation of vacuoles which swell more and faster than the rest of the cytoplasm, and in this case, unlike that of *Echinometra* eggs, fail to be discharged. Quantitative studies are needed to elucidate the nature and significance of this phenomenon.

In this as in other types of discrepancies we have to keep in mind the possible participation of electroosmotic forces.

The experiments of SKOWRON and SKOWRON (1926) on eggs of *Sphaerechinus granularis* have not been mentioned heretofore because these authors used only 7 to 14 eggs for each concentration, and the degree of scatter in the recorded volumes is so great as to destroy confidence in the calculated mean volumes. The data, after correction of arithmetical errors, suggests a possible regularity corresponding to a non-solvent volume of about 25%, but only if the obviously aberrant values for the highest and the two lowest osmotic pressures are neglected.

Summarizing the evidence as to the relation between osmotically and analytically determined non-solvent volumes we con-

clude that exact correspondence is rather exceptional: most eggs do not behave as perfect osmometers even after allowance is made for the known amount of water and solids in them. It seems necessary to conclude that much research on this topic is still needed.

In the discussion above we have tacitly assumed the cell to be homogeneous. This assumption would not in any event affect the agreement, or lack of it, between analytically and volumetrically determined non-solvent volume.

There is evidence that the osmotic volume changes of different parts of the eggs of marine invertebrates may differ in degree, as well as that the rigidity of vitelline or similar membranes may cause them to resist shrinkage, thus allowing passive volume changes of the space between them and the egg cytoplasm to take place. The latter effect is shown by *Ceratocephale* (KAMADA and YAMAMOTO 1931). The cytoplasmic nuclear volume ratios of eggs of *Sphaerechinus granularis* were studied by SKOWRON and SKOWRON (1926). These eggs were placed in hypo- or hypertonic glucose solutions and camera lucid drawings made after an arbitrary time. From these the volumes of the nuclei¹) and cytoplasm were calculated, assuming sphericity of both. The authors believe their data to show that in hypotonic media the nucleus swells relatively more than the cytoplasm and attempt to explain this by assuming the nucleus to have a higher osmotic pressure than the cytoplasm. Because of the small number of eggs studied, [7—14 at each dilution, compared with more than 300 used by McCUTCHEON, LUCKÉ and HARTLINE (1931)] and the wide scatter it might be doubted whether there is any difference in the amount of swelling of these two parts of the egg. But in view of the fact that BECK and SHAPIRO (1936) have obtained similar results with eggs of *Asterias forbesii* we may tentatively assume the validity of the findings in both cases, and surmise that the phenomenon may be of general occurrence, at least in echinoderm eggs. But the explanation offered by SKOWRON and SKOWRON (1926) appears untenable. If, as they suggest, the "osmotic pressure" in the nucleus exceeds that in the cytoplasm, this could only refer to the osmotic pressures of the two regions at equal hydro-

¹) The term nucleus is used here in a loose sense, referring to either the germinal vesicle or female pronucleus.

static pressures. In the egg itself the nucleus would have to be prevented from swelling by hydrostatic pressure due to mechanical resistance to swelling. This resistance to swelling would presumably increase as the nucleus became swollen, and hence would diminish the swelling of the nucleus. The cytoplasm not being so restrained would swell relatively more than the nucleus, and the cytoplasmic/nuclear ratio would increase. But SKOWRON and SKOWRON found that it decreased.

If, on the other hand, we attribute the greater swelling of the nucleus to the presence in it of less non-solvent material, we explain the facts. Furthermore, since on centrifugation the nucleus tends to occupy the centripetal half of an echinoderm egg, it is quite reasonable to suppose that the nucleus contains relatively more water and less of the denser protein and sterol material which are largely responsible for non-solvent volume. It is possible, of course, that electroosmosis accounts in some way for the observed swelling relationships of the nucleus and cytoplasm. We have no information as to this, but it seems unlikely that it is a major factor.

The experiments of LUCKÉ (1934) are in agreement with the above conclusion insofar as they show that the centrifugal half of a centrifuged *Arbacia* egg includes a relative excess of non-solvent material. The eggs were centrifuged in 0.75 sucrose until they pulled apart into light, colorless and heavy pigmented halves; equilibrated in sea water, and finally returned to normal sea water. Fertilization tests showed that injury, though possibly present, was certainly not excessive. The non-solvent volumes, deduced from the observed volume changes on transfer to dilute sea water, were: colorless light halves, 9.1% (these include the nuclei); pigmented heavy halves, 16.5%. The fact that the visible cytoplasmic granules, other than the oil droplets, occupy the heavy halves suggests that they are largely responsible for the non-solvent volume of the cytoplasm.

The effects of various physiological changes on equilibrium volumes. Spontaneous aging of unfertilized eggs of the sea urchin, *Arbacia punctulata*, affects their rate of swelling in diluted sea water. This is clearly shown by GOLDFORB and SCHECHTER (1932a) and GOLDFORB (1935). Much of the observed increase in rate of swelling takes place before there is evidence of injury as shown by the fertilization and early development of

the eggs. It would be interesting to know whether these changes result from or are connected with the spontaneous oxidation which LOEB and LEWIS (1902) and LOEB (1906) considered to be a lethal process in unfertilized eggs of *Arbacia* as well as in those of the starfish, *Asterias forbesii*. Unfortunately neither GOLDFORB and SCHECHTER nor GOLDFORB actually measured the equilibrium volumes of the eggs in dilute sea water, and it is impossible to say whether or not any change in driving force occurs with aging. Presumably, however, any such change should also affect the eggs in undiluted sea water, and there was no evidence of this.

On the other hand LEITCH (1936b) found that aging of eggs of *Echinometra lucunter* in sea water at 28°, (the temperature normal for this species) resulted after about 2 hours in the onset of the following parallel changes: (a) sharp decrease in the number of eggs, capable on being fertilized with fresh sperm, of developing into swimming larvae; (b) increasing transparency of the protoplasm with swelling; (c) breakdown of cell constituents resulting in an increased total of osmotically active material in the egg cell; (d) increasing degree of flattening of eggs viewed horizontally; (e) increasing non-solvent volume as calculated from the relative increase in volume in diluted sea water, and finally; (f) changing permeability to water. After a slightly longer time these processes culminate in a phenomenon resembling pale cytolysis and ending in disintegration of the egg. In this work it is very clearly shown that loss of capacity to segment and develop precedes by quite a long time the loss of the capacity to form fertilization membranes. The latter criterion is often relied upon to reveal injury, but is obviously inadequate.

While no definite interpretation of these phenomena can be made at present, we tentatively conclude that even before the capacity for fertilization is affected, aging sea urchin eggs may actually be injured, so that incipient vacuolization with loss of volume as described above for *Echinometra* may act to reduce the apparent equilibrium volumes of the eggs in diluted sea water. It is not necessary to suppose either a marked change in actual non-solvent volume (which seems inherently improbable), or a change in driving forces except as they may be effected by breakdown of egg substance. The second alternative is not however, inherently improbable.

These experiments by LEITCH throw serious doubt on many of the earlier experiments described in this section, and much of this work should be repeated with adequate attention to possible effects of progressive injury on the observed egg volumes. These might be either increased or decreased by injury depending upon the nature and sequence of the experimental procedure.

LILLIE (1918) found that neither fertilization by sperm nor artificial activation of eggs of *Arbacia* affected their equilibrium volumes. Therefore any change in their rate of swelling would presumably be due to a change in permeability to water.

The effects of various physical and chemical environmental factors on the osmotic equilibria of the eggs of marine invertebrates have been the subject of a number of studies. It should be possible, as in the case of the erythrocyte, to analyze these effects into results of penetration of ions or molecules, and changes in dissociation, activity coefficients, etc., of cell components as a result of intracellular changes in pH, temperature, and composition. But we do not yet know enough about the permeability relations of these cells nor about the nature and dissociation constants of the colloidal electrolytes within them, and a satisfying analysis has not yet been made. We can, however, note the following findings:

(a) The effects of chemical agents. Many substances have been claimed to influence the equilibrium volume of egg cells of the type here under consideration. In some cases (e. g., STEWART (1931a) it is frankly a result of an increase in intracellular solutes due to permeability to the agent used; in other cases such as FAURÉ-FREMIET's experiments (1923, 1924) the changes in volume of *Sabellaria* eggs in the common chlorides may be due entirely or in large part to secondary effects of the ions used, as the author suggests, or they may be due principally to penetration of the salts. These experiments will be considered in later chapters.

BLUMENTHAL (1927) claims to have found that HCN and KCN change the volume of *Arbacia* eggs. This may be so, but his data do not show it, although incorrect calculations and neglect to mention contrary evidence contained in the data make the conclusion appear plausible. No credence can be given to this work.

LUCKÉ (1931) has shown that the eggs of *Arbacia punctulata* are not affected as to volume in normal or diluted sea water by the addition of narcotic amounts of several urethanes or carbamates.

(b) Effects of changes in the pH of the sea water. These changes in pH are best produced by adding HCl or NaOH to the sea water; they should not be carried to a pH more than about 10.0 because above this point Mg is precipitated and additional changes in the sea water, besides those of pH, will be operative; similarly when HCl is added to sea water either the CO_2 or HCO_3 , will also differ according to the nature and amount of correction content or both will be changed, and the results of the experiment (such as removal of excess CO_2) which is applied. When other acids, such as butyric acid, are used the experiments cannot be compared with those in which HCl is used, as EPHRUSSI and NEUKOMM (1927b) for example have attempted to do: butyric acid and HCl may produce very different intracellular pH changes.¹⁾ It is also necessary for precise determinations to correct for changes in the osmotic pressure of the sea water when its pH is altered. FAURÉ-FREMIET (1924) found changes in volume of eggs of *Sabellaria* with change in pH, and EPHRUSSI and NEUKOMM (1927) found similar changes in the eggs of *Paracentrotus*.

Sea water mixed with HCl or NaOH was allowed to act upon the eggs for 45 minutes; FAURÉ-FREMIET made corrections for the slightly changed osmotic pressure. In Figure 9 the amount of water per gram of dry substance is plotted against the external pH

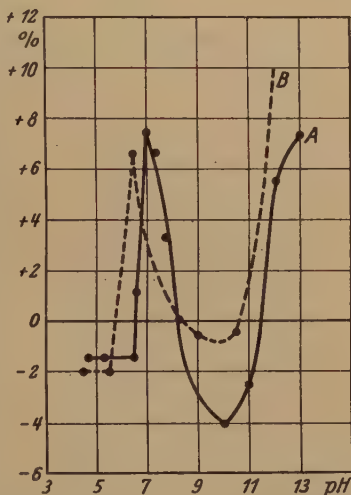


Figure 9. The amount of water per gram of dry substance is plotted against the external pH for *Sabellaria* and *Paracentrotus*.

¹⁾ See for example: M. M. BROOKS, U. S. Publ. Health Serv., Publ. Health Rep'ts 38: 1449—70 (1923).

pH for both of these cases. The important parts of these curves lie between pH 5 and 10; at lower pH values injury is visible, and at pH more than 10 both cytolysis and Mg precipitation occur. Within the range pH 5 to 10 the volumes as plotted rise rapidly from about or a little less than normal to a maximum (6 or 7% above normal) at about pH 6.5 to 7.0, and then fall off rapidly to about 4% below normal at pH 10. It might be surmised from its position that the maximum corresponded to an isoelectric point of some kind, but possible explanations are not obvious.

Experiments by LUCKÉ and McCUTCHEON (1926) on *Arbacia* eggs are in apparent disagreement with those just discussed in that no changes of volume exceeding the probable error of determination were found except under extreme conditions when there was obvious injury. The only apparent differences in procedure were that LUCKÉ and McCUTCHEON used fewer eggs than the other workers, and used sea water first "neutralized" with HCl and aerated to remove CO₂, and then brought to the desired pH. Their use of so few eggs has been criticized by EPHRUSSI and NEUKOMM, but LUCKÉ and McCUTCHEON feel that the calculated probable errors justify their use of 10 eggs for each point.¹⁾ In all probability both sets of results may be accepted, tentatively at least, as valid for the species concerned. Further studies are needed, taking into careful account possible injury where volume changes are found. LUCKÉ and McCUTCHEON (1926) also studied the results of varying the pH by the use of CO₂ or NH₄OH, which might be expected to produce greater effects on intracellular pH (See Chapter X). But these also produced no evident volume changes so long as the eggs were uninjured. EPHRUSSI and NEUKOMM (1927 b) used butyric acid to decrease the pH of sea water, but seem to have assumed that the relation between volume and pH would be like that when HCl was used (1927 a). In view of the great penetrability and toxicity of butyric acid, as noted above, such an assumption is obviously unjustifiable.

¹⁾ Histograms made for eggs of the sea urchin *Strongylocentrotus purpuratus* in certain osmotic experiments lead us to place little reliance on probable error calculations for less than one hundred individual eggs of this form. *Arbacia* eggs may be more uniform, but in any event too much reliance should not be placed on calculated probable errors for so few as ten individuals, the number used by LUCKÉ and McCUTCHEON.

(c) The effects of temperature were also studied by FAURÉ-FREMIET (1924), by EPHRUSSI and NEUKOMM (1927a, 1927b) and by LUCKÉ (1925), using as in the work on pH, eggs of *Sabellaria*, *Paracentrotus* and *Arbacia* respectively. In the first two forms there occurs a gradual decrease in egg volume from a low temperature up to about that normal for the animals. Then over a range from 18 or 19° to 24 or 25° the volumes are relatively constant, decreasing further as the temperature is raised above this range. The whole variation in egg volume is from about 3 to 4% above normal to 10 to 12% below, a change comparable for *Sabellaria* to that producible by changing the external osmolar concentration from 0.95 to 1.17 as FAURÉ-FREMIET points out. The cause of these changes has not been found. An observation by FAURÉ-FREMIET may be significant: over the range of temperatures in which no change in volume was found, there appeared and grew to maximum size with increasing temperature a large number of granules stainable by Janus Green. (These disappeared at higher temperatures.) If the process leading to appearance of the granules involved a change in the activity coefficient of intracellular water, or a liberation of bound water as some would prefer to call it, then it would be possible to account for the constancy of cell volume over that range. (The disappearance of the granules at higher temperatures could not then be simply a reversal of the process leading to their appearance.)

The work of LUCKÉ (1935) indicates that *Arbacia* eggs differ from those of *Sabellaria* and *Paracentrotus* in that they show no effect of temperature (5.4 to 29.3° C) on volume. Over this range the other two forms showed a volume change of about 8%. LUCKÉ also observed the volumes of eggs in 60% sea water at 12° and 24° and found no difference. Since neither FAURÉ-FREMIET nor EPHRUSSI and NEUKOMM appear to have tested the fertilizability of the eggs used by them, and especially since temperatures above 27° are known to prevent normal development of *Sabellaria* eggs (FAURÉ-FREMIET 1924, p. 260) one is tempted to conclude that the observed shrinkage of these eggs after 45 minutes at 30° or above is due to injury with vacuolization as described above for *Echinometra* eggs by LEITCH (1936). Volume-time curves at the various temperatures would go far towards settling this question. The reason for the occurrence of increasingly greater

swelling at temperatures below 18° would have to be sought elsewhere.

(d) **The effect of fertilization on equilibrium volume of eggs of *Arbacia punctulata*** in dilute sea water was assumed to have been negligible in LILLIE's experiments (LILLIE 1916). Apparently actual measurements were made only in the case of fertilized eggs, but the swelling curves of artificially activated and unfertilized eggs suggest that their equilibrium volumes would have been the same as those of fertilized eggs. But experiment by PAGE (1929) on the changes in susceptibility of these eggs to cytolysis in 25% sea water suggest that rapid changes in rate and amount of swelling follow fertilization. Furthermore, HOBSON (1932) gives swelling curves for eggs of *Psammechinus miliaris* which strongly suggest that when placed in 50% sea water at different times after fertilization the eggs approach different equilibrium volumes. It therefore seems probable that in general fertilization is followed by rapid fluctuations in either or both the activity of water within the cell, and the change of activity of water within the cell with change in water content. The driving force for entry of water from a given hypotonic solution cannot be assumed to be constant following fertilization. The same considerations may well apply also to artificially activated eggs.

(e) **The effect of aging of unfertilized eggs.** In somewhat the same way the driving forces for entry of water into unfertilized eggs of *Arbacia punctulata* from hypotonic sea water change with aging. GOLDFORB (1935a, 1935c) has shown that these eggs on standing in sea water gradually increase in size during 23 to 50 hours, and after this shrink with ejection of fragments and loss of capacity to cleave normally, and finally cytolysed. This suggests increase in the amount of osmotically active material in the egg, but there is no proof of it. Less easy to explain is the fact that with aging the eggs swell to relatively larger equilibrium volumes in 60% sea water, i. e., $\Delta V_{\infty} > \Delta V_0$, and $\Delta (V_{\infty} - V_0) > \Delta V_0$. The increase in $(V_{\infty} - V_0)$ may reach 12.2 to 39.6% as compared with a maximum increase in initial volume, V_0 of 5.8%. There arises, therefore, a modified response to dilution of the cell contents, either a resistance to any increase in the activity of water, or the operation of some totally different factors which are as yet unknown. Concomitant changes in consistency of the egg cytoplasm (PAGE 1929; GOLDFORB and

SCHECHTER 1932, GOLDFORB 1935b) and other properties are undoubtedly significant, but cannot yet be interpreted.

These studies like those on fertilized eggs suggest that the picture of a sea urchin egg as an elastic sac containing inert ("non-solvent") and osmotically active material, and responding passively to external osmotic changes is over-simplified. While in particular cases it may be essentially valid, it need not surprise us if in other cases processes occur in the egg cell which greatly modify its response to changes in external osmotic pressure.

The permeability of eggs of marine invertebrates to water

Special equations for calculating from the observed volume-time curves of spherical egg cells in anisotonic solutions, their absolute permeability to water, have been derived by a number of authors. (LILLIE 1916, NORTHROP 1927a, 1927b, LUCKÉ, HARTLINE and McCUTCHEON 1931, LEITCH 1936b.) These equations, their derivation and their applicability, have been critically considered by LEITCH (1936b). With the exception of LILLIE's equation (See Chapter II) all of these equations start from a differential of the type:

$$\frac{dV}{dt} = kA(\Pi_1 - \Pi_0).$$

In this equation correction has been made by LUCKÉ, HARTLINE and McCUTCHEON and by LEITCH for non-solvent volume, so that it becomes:

$$\frac{d(V-b)}{dt} = kA(\Pi_1 - \Pi_0).$$

If this equation is to be used directly without integration one may determine the $(V - b)/t$ slope graphically from a volume/time curve, insert the proper values of A and Π_1 and solve for k . If the equation is to be integrated and k found from the integral equation then one must either assume $A = A_0 = \text{constant}$, or one must transform A into a function of volume and then integrate. The latter method has been used by NORTHROP (1927b) and by LUCKÉ, HARTLINE and McCUTCHEON (1931). The former alternative has been adopted by NORTHROP (1927a) and LEITCH (1936). Some data such as those of LUCKÉ and co-workers using eggs of *Arbacia punctulata* seem to yield slightly better agreement between observed volume/time curves and those calculated from

integrated equations, when A is supposed to vary (NORTHROP 1827b) or when in addition an allowance is made for non-solvent volume (LUCKÉ, HARTLINE and McCUTCHEON 1931). But LEITCH (1936b) found that for eggs of *Echinometra lucunter* none of the equations were completely satisfactory, and that if anything, the original LILLIE equation, though it is open to criticism on theoretical grounds (NORTHROP 1927a) gave the best agreement between theory and experiment.

It is, therefore, doubtful whether any of the integrated equations have any general advantage over the simpler differential forms. For this reason they are not given here, and the interested reader is referred to the original papers. It might be concluded that the theoretical aspects of swelling, as represented by these equations, have been refined to a degree unwarranted by the present status of our experimental knowledge.

The absolute values of normal permeability to water

In Table IX we have given the available data as to the permeability of the eggs of marine invertebrates to water; recalculating in all cases so as to give P in our standard units of $\text{GM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \cdot (\text{GM} \cdot \text{l}^{-1})^{-1}$. In most cases this has involved only the use of a conversion factor, the permeability constants having been calculated in other units by the authors. In the case of the data obtained by LILLIE (1916) (Table X) we have calculated P from the original data, using equation 7 of LEITCH (1936a). A rough calculation has been made from Figure 1 in HOB-

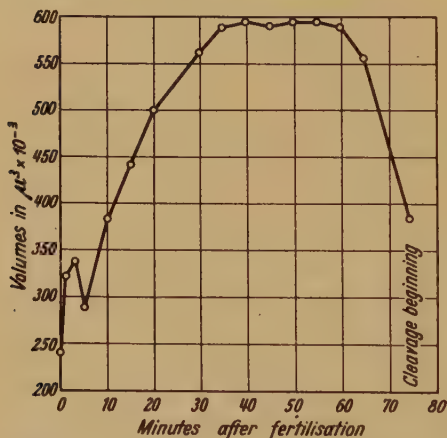


Figure 10. Variation in rate of initial water intake at various times after fertilization.

SON'S paper (1922) (Fig. 10) using the average dV/dt for the

first two minutes of swelling in the formula $P = \frac{dV}{dt} \cdot \frac{1}{S_0(a_1 - a_e)_0}$

TABLE IX. Permeability constants (P) for swelling or shrinking of presumably normal unfertilized eggs of various marine invertebrates. The direction of water transfer may be deduced from the sign of the term ($a_1 - a_0$). Bracketed values for the non-solvent volume are assumed; the others are based upon observed osmotic volume changes at "equilibrium"

Species	Initial activity difference ($a_1 - a_e$) GM/l-1	Temperature °C	Non-solvent volume % of V_0	Permeability Constants, $P \times 10^{-8}$ GM/cm ² /sec ⁻¹ / (GM/l-1) ⁻¹	Authors
<i>Arbacia punctulata</i>	+0.59	—	(12)	2.8 — 4.2	5
"	+0.59	12	(0)	1.04	7
"	+0.39	18	(12)	1.70	6
"	-0.39	18	(12)	1.97	6
<i>Strongylocentrotus purpuratus</i>	+0.40 — 0.60	18	24.3	1.33 — 2.16	3
" <i>franciscanus</i>	+0.40 — 0.60	18	18.0	2.41 — 2.84	3
<i>Echinometra lucunter</i>	+0.32 — 0.54	28	34.0	5.12 — 13.52	4
<i>Psammecchinus miliaris</i>	+0.50 (approx.)	18	(0)	5.0 (approx)	1
<i>Dendraster excentricus</i>	+0.40 — 0.60	21	(15)	2.66 — 3.07	2
<i>Patiria miniata</i>	+0.40 — 0.50	21	(15)	1.52 — 2.06	2
<i>Pisaster ochraceus</i>	+0.40 — 0.70	21	0	6.08 — 9.45	3
<i>Urechis caupo</i>	+0.40 — 0.60	21	48	3.84 — 5.57	3

References: 1. HOBSON (1932) 2. LEITCH (1931) 3. LEITCH (1934) 4. LEITCH (1936b) 5. LILLIE (1916)
6. LUCKÉ, HARTLINE and McCUTCHEON (1931) 7. McCUTCHEON and LUCKÉ (1928).

HOBSON, A. D., J. Exp. Biol. 9: 69—92 (1932). — LEITCH, J. L., Univ. Calif. Publ., Zool. 36: 127—40 (1931). —
LEITCH, J. L., J. Cell. Comp. Physiol. 4: 457—73 (1934).

which yields the tabulated approximate value for unfertilized eggs of *Psammechinus miliaris*. Table XI gives values for a number of eggs recalculated from data of LEITCH (1934). It will be seen that all the values are of the order of magnitude 10^{-7} to 10^{-6} ; at comparable temperatures most of the values lie close to 2×10^{-7} . Exceptions are *Urechis*, the only species not an echinoderm, and *Pisaster* in which the osmotically determined non-solvent volume of 0 is undoubtedly incorrect, since the analytically determined value is about 20%. The use of the higher figure would not greatly affect the value of P which would still be higher than most of the other values.

The greater permeability to water of *Arbacia* eggs outwards as compared with inwards calls for comment. These measurements concern eggs swollen in 60% sea water and then trans-

Table X

Time in Sec.	$V \text{ cm}^3$	$V - b$	$P = (\text{GM} \cdot \text{cm}^{-2} \text{sec}^{-1} / (\text{GM} \cdot \text{l}^{-1})^{-1})$
0	21.3×10^{-8}	18.8×10^{-8}	indeterminate
60	22.9	20.4	4.16×10^{-7}
120	24.5	22.0	4.175
180	26.1	23.6	4.145
240	27.9	25.4	3.99
300	29.2	26.7	3.82
360	30.7	28.2	4.065
420	31.3	28.8	3.822
480	32.0	29.5	3.480
540	32.7	30.2	3.285
600	33.6	31.1	3.20
660	34.6	32.1	3.145
720	35.1	32.6	2.995
780	35.8	33.3	2.90
840	36.3	33.8	2.790
	40.4	37.9	

Assume $b = 12\%$ of V_0 ; $S = \text{const.} = 1.716 \times 10^{-4} \text{ cm}^2$

$V_m = 18 \text{ cc.} = 18 \text{ cm}^3$.

$(\Delta IT)_0 = 11 \text{ atm. (after LILLIE, 1916)} = 0.46 \text{ GM.}$

Recalculation by S. C. BROOKS of LILLIE's results (1916) on *Arbacia* eggs, using Equation 7 of LEITCH (1936).

ferred to 100% sea water in which they shrank again to very nearly their original volumes. (LUCKÉ, HARTLINE and McCUTCHEON 1931.)¹⁾ Whether this difference is due to the unidirectional operation of non-osmotic forces or to some as yet unknown factors must be left for future study. Similar differences in permeability to inflowing and outflowing water are often noted, although they have not been accurately measured.

Table XI. Effects of change in concentration of sea water on swelling of invertebrate eggs. From LEITCH 1931 and 1934.

Species	$P \times 10^{-7}$ at initial activity differences in GM · liter ⁻¹			
	0.70	0.60	0.50	0.40
Pisaster ochraceus	3.39	3.86	2.93	4.55
	7.04	8.01	6.08	9.45
Strongylocentrotus franciscanus	—	1.37	1.33	1.16
		2.84	2.76	2.41
S. purpuratus	—	1.04	0.80	0.64
		2.16	1.66	1.33
Urechis caupo	—	2.68	1.85	1.98
		5.57	3.84	4.12
Dendraster excentricus . . .	—	1.28	1.31	1.48
	With jelly	2.66	2.72	3.07
With jelly removed	—	0.83	1.14	1.59
		1.72	2.37	3.30
Patiria miniata	—	—	0.73	0.99
			1.52	2.06

The relative permeability of the eggs of *Arbacia punctulata* to "heavy water" has been studied by LUCKÉ and HARVEY (1925). No other species appears to have been used in this way

¹⁾ The use of the LILLIE equation had previously led McCUTCHEON and LUCKÉ (1926) to conclude that permeability to water was affected by external osmotic pressure, but in the present paper the use of a more appropriate equation shows that permeability to water is practically independent of any but the most extreme changes in external osmotic pressure. (See Table X.)

as yet. The eggs were first allowed to come to equilibrium in natural sea water or in artificial sea water containing 0, 0.2 or 99.5% D_2O , which was then diluted by addition of the corresponding type of water, i. e., pure H_2O , or H_2O containing 0.2 or 99.5% D_2O . All values fell between 0.106 and 0.110 in their units, or between 2.20 and 2.28 $\text{GM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \cdot (\text{GM} \cdot \text{l}^{-1})^{-1}$. Thus, H_2O and D_2O being present in the same ratio in the cell and its surrounding medium it is clearly shown that there is little or no difference in the permeability of *Arbacia* eggs to D_2O as compared with H_2O . (See also the discussion of this point with respect to erythrocytes.)

Experimental modification of the permeability to water of the eggs of marine invertebrates

While normal unfertilized eggs of marine invertebrates may ordinarily act as passive osmometers, at least during the initial states of swelling, it cannot safely be assumed that such is the case when conditions other than osmotic pressure are changed. All the results mentioned below must be regarded as subject to re-examination in this light. The factors studied include temperature, non-electrolytes, narcotics, anaerobiosis, valency and nature of ions, injury fertilization, artificial activation and aging. The effects of pH on permeability of these eggs to water appear to have been neglected. All the experiments refer to *Arbacia punctulata* except as otherwise noted.

(a) The effects of temperature on permeability to water have been studied by McCUTCHEON and LUCKÉ (1926, 1927). These two papers deal with endosmosis and exosmosis respectively. In both cases a Q_{10} of between 2 and 3 was found. The results are also stated of μ , the constant of the Arrhenius equation, and these values lie between 13,000 and 19,000. Since μ here represents a critical increment of energy per molecule, objection may legitimately be made to the propriety of transferring this concept to permeability unless or until it can also be shown that a molecular activation is necessary for the passage of a water molecule across the plasma membrane.

The Q_{10} values of 2 and 3 are to be compared with the value of 1.3 reported by JACOBS (1928) for hypotonic hemolysis.

In a subsequent paper LUCKÉ and McCUTCHEON (1930) report that a few minutes exposure of *Arbacia* eggs in sea water

to temperatures of 39 to 41° markedly increases their permeability to water, and that such eggs on subsequent return to room temperature and fertilization fail to cleave normally. More intense injury may induce shrinkage and cytolysis following the first few minutes of swelling. These are obvious injury effects.

In the same paper it is shown that even very moderate temperatures (18°) acting in conjunction with hypotonicity (50% in sea water) may produce distinct increases in subsequent permeability to water. No such effect occurred at 14°. We are inclined to attribute the large value for the temperature coefficient of permeability previously found by these workers and given above, to injury produced by the combined action of moderate temperatures ($> 15^{\circ}\text{C}$) and hypotonicity (40% sea water) in part at least. This question should be re-examined.

(b) The effects of non-electrolytes are probably to be thought of as due in some part at least to the absence of ions, since no attempt has been made to change the concentration of such substances in the medium in which meanwhile the ionic strength and osmotic pressure are kept unchanged¹). Thus McCUTCHEON and LUCKÉ (1928) found the permeability to water of eggs placed in solutions of sucrose, glucose or glycine isotonic with 40% sea water to be greater than in the latter; the values given are 2.95, 2.03, and 2.14×10^{-7} respectively for the non-electrolytes, as compared with 1.04×10^{-7} for dilute sea water. Even though this may be an effect of lack of ions attention may be called to the fact that glucose and sucrose accelerate the diffusion of urea through a gelatin gel, no strong electrolytes being present (FRIEDMAN 1930). The parallelism is suggestive.

(c) The effects of narcotics on unfertilized eggs were studied by LUCKÉ (1931) using several urethanes and carbamates. The addition of these substances to sea water had no effect on either equilibrium volumes or permeability, but when they were added to glucose solutions the rate of entry of water was reduced, but not to the low level characteristic of sea water solutions or

¹) Ionic strength is defined by LEWIS and RANDALL (1921) as $\mu = \frac{1}{2} \sum m_i z_i^2$ where m_i and z_i refer to the concentration and valency of each ion present. This measures many of the physiological properties of solutions containing ions more or less irrespective of the nature of the ions.

the similar low level found in glucose solutions plus 0.01 CaCl_2 . Unfortunately LUCKÉ did not determine whether narcotics when used in the absence of Ca affect equilibrium volumes. If they do not, then LUCKÉ's conclusion that these narcotics lower permeability, although this effect may be masked or prevented by Ca, may be accepted.

The earlier work of LILLIE (1918) was concerned primarily with the prevention by narcotics of the increase in permeability which follows fertilization. Using the lower alcohols, ethyl ether, chloroform, chloral hydrate and ethyl urethane in concentration sufficient to stop cleavage he found that the rate of shrinkage of fertilized eggs of *Arbacia punctulata* in hypertonic sea water was kept about like that of unfertilized eggs, while unnarcotized control eggs shrank more rapidly. Cyanide was ineffective. In view of some reasonable doubt as to the combined effects of narcotics and fertilization on the driving forces for water it cannot be regarded as proved that this is an effect on permeability; but such an effect seems probable.

It is difficult to reconcile the results of LUCKÉ and of LILLIE with those of HEILBRUNN (1925), who found that ether in the concentrations in sea water used by LILLIE increased the rate of swelling of *Arbacia* eggs when the solution was diluted. LUCKÉ suggests that injury occurred in HEILBRUNN's experiments (see below), but this cannot be accepted as proved. Further work is needed, and may well show that ether, and perhaps other narcotics, are capable of producing opposite effects dependent upon concentration, time of action, presence or absence of Ca, etc.

Attention should be called to ANSELMINO's finding (1928) that narcotics decrease the permeability of non-living membranes to water.

The effects of preventing normal oxidations by CN or anaerobiosis would appear to be small. LILLIE (1918) found that while M/8000 KCN stopped cleavage, a concentration of at least M/800 was necessary before any retardation of exosmosis of water from fertilized eggs could be detected. KEKWICK and HARVEY (1934) subjected unfertilized *Arbacia* eggs to anaerobiosis in an atmosphere of hydrogen and found that it led to a small but definite decrease in swelling rate with no effect on the equilibrium volumes.

The effects of ions on permeability of *Arbacia* eggs to water have also been studied by McCUTCHEON and LUCKÉ (1928), who

found that the rate of swelling at the second minute $\left(\frac{dV}{dt} \cdot \frac{1}{S(a_0 - a_1)}\right)$ in a hypotonic glucose solution was increased by Na and possibly K, and decreased by Ca and Mg. The chlorides were used and in each case the resulting solution was 0.04 M with respect to the salt (Table XII).

These permeabilities are calculated by a formula which takes no account of possible changes in equilibrium volume (driving force) and no determinations of equilibrium volumes were made. With these reservations it seems probable that cations have the capacity to decrease permeability to water and that this action increases with increase of valency. On this basis we would need

Table XII. The permeability of eggs of *Arbacia punctulata* to water in various solutions isoosmotic with 40% sea water. (After McCUTCHEON and LUCKÉ 1928.)

Solution	Permeability, $\text{GM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} (\text{GM l}^{-1})^{-1}$
Glucose 0.40 M	1.93×10^{-7}
" " " + NaCl 0.04 M . . .	$2.68 \times$ "
" " " + KCl " " . . .	$1.99 \times$ "
" " " + CaCl_2 " " . . .	$1.12 \times$ "
" " " + MgCl_2 " " . . .	$1.04 \times$ "
Sea water 40 + tap water 60 parts .	$1.00 \times$ "

to suppose that the effect of the anion Cl is to increase permeability and that this effect exceeds that of Na and about equals that of K. Alternatively we would have to suppose that Na, but probably not K, increases permeability to water. This point is further discussed in Chapter XII.

The effect of sign and valence of ions has been studied further by LUCKÉ and McCUTCHEON (1929) using similar methods and material. The salts used were cobaltamine chlorides whose cation valencies were 0, 1, 2, 4 and 6, and sodium chloride, sulfate, citrate and ferrocyanide (anion valencies 1, 2, 3 and 4). Exceedingly low concentrations were used. These salts decreased permeability more, the greater the cation valency, and increased permeability more, the greater the anion valency, thus confirming the interpretation of the earlier experiments as given below.

(d) The effects of fertilization on permeability to water were the primary concern of LILLIE, whose pioneer investigations underlie nearly all the work on permeability to water so far reviewed. LILLIE (1916) studied the rate of swelling of *Arbacia* eggs, unfertilized and after fertilization or artificial activation, when placed in dilute sea water. Recalculating his data to standard units the observed permeabilities of those eggs were 2.8 to 4.2, 9.14 to 9.50 and 7.55 to 7.58 $\times 10^{-7}$ GM \cdot cm $^{-2} \cdot$ sec $^{-1}$. (GM \cdot l $^{-1}$) $^{-1}$. Either method of initiating cleavage thus led to an increase in the permeability of the egg to water. LILLIE (1918) supplemented these experiments by others in which the eggs were watched during shrinkage in a hypertonic medium, or during swelling on subsequent return to sea water. The results are in agreement with the earlier work, but are not capable of yielding accurate quantitative results because the eggs undergo crenation.

A more detailed study has been made by HOBSON (1932) using eggs of *Psammechinus miliaris*. Figure 8 taken from his paper shows how the rate of initial water intake from 50% sea water varies with time after fertilization. Much difficulty was encountered because of varying susceptibility to cytolysis, but the time/volume curves in this paper strongly suggest that the equilibrium volumes approached by the eggs also varied greatly with time after fertilization. If this was true, the driving force changed continuously after fertilization. Add to this the fact that either or both driving force and permeability are changing continuously during the swelling process, and one sees that the situation is exceeding complex. HOBSON is much to be commended for having deliberately refrained from stating his results in terms of permeability "constants".

In the light of HOBSON's experiments it may well be necessary to revise the conclusion usually drawn from LILLIE's experiments. It is certain that the rate of water intake is affected by fertilization and the ensuing processes in the sea-urchin eggs studied, but this may be due to one factor: change in driving force and change in permeability.

(e) The effects of aging have been discussed by GOLDFORB (1935) and the effect on permeability to water deduced from the rate of swelling at the third minute:
$$P = \frac{dV}{dt} \cdot \frac{1}{S(a_0 - a_1)}$$
 Here again equilibrium volumes were not studied and we cannot

exclude driving force as a factor in causing the increase in rate of swelling with aging. During the first few hours after shedding this increase is slight, but in 36 to 60 hours it becomes marked. Injury as shown by the proportion of eggs incapable of cleavage when fertilized seems to be later in appearing, but more rapid in its spread than the permeability change, and GOLDFORB considers the two phenomena independent. But the former is dependent upon variability distribution, while the latter is a property of each individual egg measured. It can hardly be argued that lack of parallelism of the time curves for the two processes shows them to be independent of each other; but in any event that one of the two factors governing the rate of entry of water from hypotonic solutions, i. e., either driving force or permeability increases slightly with aging in the case of *Arbacia* eggs in sea water.

The permeability of the nuclear membranes of echinoderm eggs to water has not been successfully evaluated. An approach has been attempted by BECK and SHAPIRO (1936), who have shown that although the germinal vesicles of eggs of the starfish, *Asterias forbesii*, swell proportionately more than the cytoplasm when the eggs are put into hypotonic sea water, they do so more slowly. Theoretically this must result in part from the initial lack and subsequent appearance and decline of an osmotic gradient across the nuclear membrane as water gradually dilutes the cytoplasm; this would give a sigmoid volume/time curve for the nucleus, a result which is vaguely suggested by the observed curve. The authors do not attempt further analysis and regard the evidence as inadequate for determination of the permeability of the nuclear membranes of these eggs. Their permeability to dyes appears to be very great (MONNE 1935), for which see Chapter XIII, and it may well be that they are also very freely permeable to water.

CHAPTER V

THE OSMOTIC RELATIONS OF THE EGGS OF VERTEBRATE ANIMALS

Very little is known as to the quantitative aspects of the permeability to water of the eggs of vertebrates. They are in general much less favorable material than the eggs of marine invertebrates, both because their larger size is due in great part to relatively inert yolk and because of the presence of relatively rigid external membranes or shells. Mammalian eggs lack these handicaps but are hard to obtain and to keep alive in vitro. Nevertheless, enough has been done with some materials to show that the field is promising. We shall mention experiments on the eggs of fish, amphibia, and a bird.

Fish eggs may be divided into two groups according to their normal surrounding medium; fresh water or sea water. But there seems to be no essential difference between the two types. In both there is the yolk or egg cell proper, surrounded by a thin (ca. $100\ \mu$) tough fibrous membrane or chorion of ovarian origin. (GRAY 1932). The yolk is considered to have on its surface a vitelline membrane whose presence is inferred from the permeability relations: it would correspond to a plasma membrane. Between it and the chorion there is more or less perivitelline fluid.

It has been shown for several species of trout and salmon (*Salmo* spp.) that the fertilized eggs in fresh water maintain an osmotic pressure not much inferior to that of the serum of the fish (GRAY 1920, RUNNSTRÖM 1920, SCHMIDT-NIELSEN and SCHMIDT-NIELSEN 1923, SVETLOV 1929). For example, in the case of *Salmo salvelinus* the freezing point of the blood serum was -0.636° ; eggs in the oviduct, -0.645° ; fertilized eggs in fresh water, -0.599° (RUNNSTRÖM 1920). These figures refer to crushed whole eggs: SVETLOV (1929) showed that the freezing

point of the yolk (*Salmo fario*) was independent of the salt content of the surrounding medium, while that of the perivitelline fluid approximately equalled that of the surrounding medium, which was experimentally varied. Furthermore, the chorion has been found to be freely permeable to water, electrolytes, sugars and many of the smaller dye molecules. (RUNNSTRÖM 1920; GRAY 1920, 1932; SVETLOV 1829; BOGUCKI 1930). At the same time the vitelline membrane could be shown to be impermeable to salts and water, so long as the egg was alive. It is particularly to be noted that there is no apparent change of volume of the yolk with change in osmotic pressure of the medium, and therefore also of the perivitelline fluid bathing the yolk. This would seem to necessitate the conclusion that the vitelline membranes of these eggs are impermeable to water. The only alternative interpretations appear to be a greatly retarded diffusion within the yolk, so that its surface layers only would respond to osmotic effects in any reasonable time, or an active regulation of its own osmotic pressure. The former would correspond to the situation found in the hen's egg (p. 000) and the latter is the view advocated by SVETLOV. None of the three interpretations can be regarded as either excluded or established.

SCHMIDT-NIELSON, AAS, ASTAD and LEONARDBSEN (1933) found a similar situation in the eggs of *Raja* spp. insofar as the osmotic pressure of the yolk was high ($\Delta = -2.90 - 3.05^\circ$) while that of the whites was lower and about equal to that of the blood serum of *Raja* and that of sea water ($\Delta = -2.08 - 2.26^\circ$). Further studies on these eggs would be interesting.

YAGLE (1930) has tried to study the permeability to water of the membranes of the eggs of *Fundulus heteroclitus*, using a method due to LOEB (1912). LOEB had shown that *Fundulus* eggs were temporarily buoyed up by concentrated salt solutions, but after a time sank, presumably because water had been withdrawn osmotically from the egg which thus became more dense. It was assumed that since shrinkage was always observed when the egg sank and never when it still floated, therefore passage of salt into the egg was negligible. The interval elapsing before the egg began to sink would, therefore, measure the time required for the exosmosis of a given amount of water and would be inversely proportional to the rate of water transfer. By this method LOEB could show that while eggs floated less than three hours

in 3 M NaCl and less than half an hour in 1.25 M CaCl_2 they floated more than three days in an appropriate mixture of the two. Hence he reasoned that antagonism between Na and Ca in this case rested upon the fact that a mixture of the two caused or maintained a high degree of impermeability of the membranes to water.

YAGLE's paper (1930), although much vitiated by misconceptions and faulty analysis, confirms LOEB in general. In addition the author reports progressive changes in flotation time, and experiments comparing 8 day embryos with and without the chorion.

Fertilization appears to increase the permeability to water just as it appears to do in the case of *Arbacia* eggs. (See above.) The parallel is superficial since in the latter case permeability has not been followed beyond the first cleavage, whereas in the former it was not tested during the interval between fertilization and the stage of embryonic shield (1 day), i. e., after many cleavages. Nevertheless the increased facility of water exchange probably represents increased permeability, and its rise during the stage of increasing growth rate and fall as the embryo approaches complete development is very suggestive.

The removal of the chorion from 8 day embryos led to results difficult to interpret, and possibly not reliable. The number of embryos so studied was small and neither their number nor the range of variation are given. It does not necessarily follow, as the author states, that because the naked embryo floats (e. g., in an NaCl solution) a shorter time than the whole egg, therefore the vitelline membrane is more permeable to water than the chorion. Even a mere mechanical barrier to convection would retard sinking, and the chorion would have this effect even if it were, as seems probable, relatively very permeable to water. As to the effects of various salt combinations, we believe the interpretation should differ in some respects from that given by the author, but as any interpretation must be speculative we shall not discuss this point.

In general it must be admitted that the results of both LOEB and YAGLE could be explained equally well by supposing the vitelline membrane to be normally impermeable to water, but to become so upon injury by unbalanced salt solutions. The application of new techniques to the study of fish eggs, such as those of *Salmo* sp., *Fundulus* may necessitate extensive revision of present concepts.

Amphibian eggs, normally found in fresh water or similar media, have been made the subject of osmotic studies by OVERTON (1899), who used fertilized eggs and cleavage stages of *Rana temporaria*, *Bufo variabilis* and *Bombinator igneus* in experiments designed primarily to test their permeability to solutes. They were found to shrink in hypertonic sucrose or NaCl solutions, but quantitative data on neither the permeability nor the equilibrium volume relations are given. BACKMANN (1912) similarly gives volume/time curves for amphibian eggs and embryos in solutions of various osmotic pressures. The points are too few to give any information as to permeability, and only a rough qualitative idea of the volume/osmotic pressure relation. FAURÉ-FREMIET (1921) has given analyses of eggs of *Rana temporaria* from which the non-solvent volume would appear to be about 42%, and TERROINE and BARTHELMEY in the printed discussion of this paper (pp. 481—82) give a similar value. It appears that these eggs, like those of fish, have an osmotic pressure, when first formed in the ovary, equal to that of the mother's blood serum. (BACKMANN and RUNNSTRÖM 1912, BIALASZEWICZ 1912). But unlike fish eggs, the eggs of such forms as *Rana temporaria*, *R. esculenta*, *Bufo* sp., and *Triton* sp. abruptly change their osmotic pressure when brought into contact with fresh water at the time of laying. This was at first regarded as an effect of fertilization (BACKMANN and RUNNSTRÖM 1912, BACKMANN 1912), but PRZYFIECKI (1917) showed that fertilized and unfertilized eggs both showed this drop in osmotic pressure. The freezing point depression of eggs after 2 to 3 hours in tap water varied between 0.05° and 0.15° regardless of whether they had been fertilized or not, while that of ovarian eggs usually has been found to be about 0.45° to 0.50° .

During development the osmotic pressure of the embryo gradually rises (BACKMANN and RUNNSTRÖM 1912) and this rise is dependent upon the presence of oxygen (BACKMANN, SUNDBERG and JANSSON 1914a, 1914b) and upon temperature (BACKMANN 1914) but the way in which oxidation leads to the osmotic pressure changes has not been determined.

It seems as though with care it might be possible to obtain adequate data on the permeability of amphibian eggs to water. It would, however, be necessary to know whether or not the yolk was homogeneous throughout, i. e., whether diffusion of water in the yolk was fast enough to be neglected.

Osmotic relations of hens' eggs. The hen's egg is an extreme example of the yolk-rich type of egg cell. The yolk of the hens' egg is generally spoken of as a single cell, but it may be seriously doubted whether the vitelline membrane which separates it from the egg white is in reality to be considered as protoplasm or bounded by a plasma membrane; only around the germinal vesicle is there a little fairly normal cytoplasm. GRAY (1932) suggests that the so called vitelline membrane of the hens' egg may well be considered to be homologous with the chorion of the fish egg; both are of ovarian origin. There has been no quantitative measure of the permeability of this vitelline membrane to water. In fact it has been supposed that it was impermeable. Early observations indicated that the osmotic pressure of the egg yolk was steadily maintained much higher than that of the egg white¹). The magnitude and possible causes of this condition were studied by STRAUB (1929), who suggested that the vitelline membrane might be the seat of oxidative processes giving rise to electrical forces which were capable of doing osmotic work on the water of the egg. But the vitelline membrane cannot support a measurable hydrostatic pressure, and the work of NEEDHAM and his collaborators casts serious doubt upon any reasonable physical explanation of the supposed difference in osmotic pressure. (SMITH and SHEPHERD 1931a, 1931b, NEEDHAM, STEPHENSON and NEEDHAM 1931, and NEEDHAM 1931). The existence of such a difference was affirmed by some and denied by others, but ultimately it was shown by HOWARD (1932) and BALDES (1934) that there is little if any osmotic gradient across the vitelline membrane of the hens' egg. The apparent difference was due to neglect of the very slowly equilibrated inhomogeneity of the yolk, whose central osmotic pressure much exceeds that at its periphery. As has been suggested above, it may possibly be that similar inhomogeneities affect the data on other eggs. The fact that diffusion of electrolytes from killed eggs or mixed yolk material is very rapid as found by GRAY (1932) working with trout eggs suggests strongly that this factor is unimportant for fish eggs at least. But it is still possible that in the intact egg cell diffusion is slower, and that the freely diffusible electrolytes which GRAY

¹) For references to the earlier work use J. NEEDHAM and M. SMITH, *J. Exp. Biol.* 8, 286—92 (1931).

found to be inadequate to account for the freezing point depression of the yolk, are in the intact egg immobilized by being bound to larger molecules. We cannot be dogmatic about conditions in yolk-bearing egg cells while this possibility remains unstudied.

Spermatozoa contain relatively large amounts of nuclear and other specialized types of protoplasm, and it has even been suggested that the chromatin in certain spermatozoa (*Sepia officinalis*) is in the form of liquid crystals (RINNE 1930). It would, therefore, not be surprising to find the osmotically induced volume changes of these cells rather different from those of more typical cells. As a matter of fact some types of spermatozoa, notably those of crabs and closely related forms exhibit most peculiar behavior: the nuclear material, which forms a cup-shaped mass partially enclosing the cell, swells little if at all in hypotonic solutions; while the portion known as the "cavity of the capsule" (BINFORD 1913) or "primary vesicle" (FASTEN 1921) increases enormously in volume, and is apparently squeezed out of the relatively firm nuclear cup. This material was supposed by BINFORD to originate during spermatogenesis as a vacuole in the cytoplasm, but there is no good evidence as to its physical consistency — fluid or gel — nor any good evidence for the chitinous nature of the "membrane" around it.

That the peculiar localized swelling of decapod sperm is in reality of general occurrence might be deduced from KOLTZOFF's extensive studies on a wide variety of form (KOLTZOFF 1908). All of these are said to "lift off a vesicle bounded by a plasm membrane" when placed in hypotonic glycerine or urea solutions. Meanwhile the nucleus, which KOLTZOFF supposes to be constrained by a "skeleton", does not increase in size to any measurable extent. If the nuclear material in these cases contains very much non-solvent substance, then even in the absence of a constraining "skeleton" very little swelling would be expected.

Measurements of the total volume of spermatozoa, analogous to those customary in the case of erythrocytes, ought then to yield results intermediate between those applicable to the nuclear material and those applicable to the portion of the cells which swells. Thus HAMBURGER found that both ripe and unripe frog spermatozoa in hypotonic and hypertonic NaCl solutions, behaved roughly like perfect osmometers provided 71.6 to 72.8 and 73.2 to 79.4% of their respective volumes could be considered

as non-solvent space (HAMBURGER 1898). Practically the entire nucleus is probably to be so considered.

Therefore, spermatozoa, even though not microscopically uniform in their swelling and shrinkage are found when studied quantitatively to obey osmotic laws to the same extent as other cells whose lack of homogeneity is not apparent to the eye.

No effect of pH on the swelling (or "explosion" as it is called) of decapod spermatozoa was detected by SUSAETA (1927) within the limits: pH 4.0 to 11.0; outside of these limits he was presumably dealing with dead or moribund material. This corresponds with GRAY's findings for trout eggs and those of LUCKÉ and McCUTCHEON for eggs of *Arbacia*, but is not like those of FAURÉ-FREMIET with *Sabellaria* nor EPHRUSSI and NEUKOMM with *Paracentrotus*.

An apparent contradiction appears when we contrast the large non-solvent volumes (and, therefore, small osmotic volume changes) of sperm which consist so largely of nuclear material, with the fact that the nuclei of echinoderm eggs swell relatively more than the cytoplasm when the eggs are put into hypotonic media. We can only guess that the non-solvent nuclear material is selectively retained in spermatozoa, so that it forms a relatively much larger fraction of the nuclear volume than it does in the relatively large germinal vesicles of echinoderme.

CHAPTER VI

OSMOTIC EQUILIBRIUM IN OTHER ORGANISMS AND CELLS

Osmotic Equilibrium in Protozoa. Although protozoa are animal cells not united except in loose colonies, and floating or swimming free in the medium just as do the erythrocytes, marine eggs, or spermatozoa considered above, nevertheless they are in reality very different. There is a high degree of differentiation of structure and function within the cell. The structures presumably most important for our present consideration include the relatively rigid cuticle possessed by many forms, and possibly also the ectoplasm¹), and the food and contractile vacuoles, both of which appear to be bounded by semipermeable surfaces. The rigid outer layers prevent swelling in hypotonic solutions, but are not sufficiently rigid and permeable to resist the water-withdrawing tendency of hypertonic solutions.

For these reasons there are almost no published data on the quantitative relations between osmotic pressure and cell volume in protozoa. CHALKLEY (1930) studied species of *Amoeba*, a particularly favorable form, determining their volumes in hypertonic glycerol, lactose, or urea solutions of 0.10 to 0.275 M. The volumes were found by calculation from the size of vertically and horizontally projected images, or by measurement of animals drawn into a capillary tube so that they were essentially cylindrical. Unfortunately data for different concentrations were obtained only in the case of glycerol, which presumably penetrated into the animals with some ease. Taking the average volume in the culture medium (hay infusion) as 100%, the losses in volume

¹) MAST (1926) considers the plasmalemma (cuticle) and the plasma-gel, which includes ectoplasm or ectosarc and the outer portion of the endoplasm or endosarc, to be semi-permeable.

observed were, 0.10 M: 19%; 0.20 M: 29%; 0.275 M: 49%. Higher concentrations were rather quickly fatal. Lactose and urea, 0.15 M, withdrew more water than the same concentration of glycerol. There is only a very rough reciprocal relation between osmotic pressure and cell volume, and the data do not permit the calculation of non-solvent volumes. Apparently a much larger body of data must be amassed before conclusions as to the water equilibrium can be reached, except on the basis of analogy with other types of cells.

CHALKLEY experimented also upon the effects of pH, using unspecified buffers in a culture fluid consisting of NaCl, KCl and CaCl_2 in concentrations of 10, 3, and 1.8×10^{-5} M respectively. Four pH values, 6.0, 6.6, 7.0 and 8.0 yielded different volumes with a minimum at 7.0. The observed volumes may quite well be a result of the swelling and shrinkage of a colloid isoelectric at or near pH 7.0. But such an explanation is probably too simple, and a more exact and extensive study is desirable. Several authors have reported shrinkage of protozoa exposed to concentrated solutions. YASUDA (1900) noted rounding up of species of *Euglena*, *Colpoda*, *Chilomonas*, *Mallomonas*, and *Paramecium*, which were placed in solutions of salts, glycerol or sugars. This might, however, be interpreted as a response to stimulus rather than actual shrinkage. MASSART (1889) reports shrinkage and deformation of *Glaucoma*, *Chilodon* and *Vorticella*; moreover *Polytoma uvella* which is surrounded by a rigid sheath was observed to be "plasmolysed" in 0.10 M KNO_3 , i. e., the protoplasmic mass shrunk away from the sheath. Similar "plasmolysis" occurred when cysts of *Vorticella* or *Colpoda* were placed in 0.2 to 0.4 M KNO_3 .

In studying osmotic water relations of protozoa we are immediately confronted with the almost universal presence of an indubitably active secretory mechanism: in the contractile vacuole. This surely plays an important rôle which makes the protozoa no longer the passive plaything of its osmotic environment. It may merely be noted here that in the case of *Amoeba* a sufficient increase of osmotic pressure in the outside medium slows up the rate of discharge of fluid or even makes the contractile vacuoles disappear (FINLEY 1930); while on the other hand, the microinjection of distilled water increases the rate of excretion of fluid (HOWLAND and POLLACK 1927). This makes

it evident that contractile vacuoles are subject to regulation by osmotic forces, but does not furnish any basis for a quantitative estimate of the osmotic work which they can perform. Moreover, contractile vacuoles may be made to appear by increasing the osmotic pressure surrounding a cell. Thus YASUDA (l. c.) reports that the contractile vacuoles of the protozoa he studied, increased in size with increasing concentration of the suspending medium. MASSART (l. c.) describes the appearance, enlargement, diminution and disappearance of contractile vacuoles in the cysts of the protozoa studied. We have evidently to deal with a complicated mechanism which should repay careful study.

Osmotic equilibria of miscellaneous isolated animal cells. Several types of animal cells are said by OVERTON (1899) to be amenable to experimentation on the changes in their volumes with change in the external osmotic pressure. These cells, which according to OVERTON, are characterized by the presence of vacuoles, include chorda cells of tadpoles and embryo fish, cartilage cells, especially those from the crystalline style of certain prosobranch molluscs, connective tissue cells, testa cells of tunicates, entoderm cells of coelenterates, and oftentimes "hydropic" tumor cells. Most of this material is extremely difficult to use, and no one but OVERTON appears to have had the courage to undertake experiments with them. No doubt careful experiments would lead to interesting conclusions: chorda and cartilage cells which can be made to shrink within their matrix, just as do plant cells within their enclosing cell walls. OVERTON found that many of these cells retain constant volume in isoosmotic solutions of various electrolytes and non-electrolytes, but shrink slowly in more concentrated solutions. Since he was primarily interested in the relative penetrability of different solutes, he gives no data usable in the present connection.

Osmotic equilibria of leucocytes were studied by HAMBURGER (1898) but unlike his work on erythrocytes, this study did not lead to further investigations. He found the volume changes of leucocytes to be quantitatively much the same as those of erythrocytes of the same species (horse). Both diluted serum and NaCl solutions were used. Table XIII is from a representative experiment, and shows the calculated values of the non-solvent volume ("festes Gerüst"). It will be observed that as with erythrocytes the swelling in the more dilute solutions is

Table XIII. The relative volumes of horse leucocytes in NaCl solutions of different concentrations, and the non-solvent volumes calculated therefrom.

(After HAMBURGER (1898), Table V.)

Observation	NaCl Concentration %	Relative Volume	Observations used	Non-solvent Calculated %	Mean non-solvent volume (HAMBURGER)
a	0.25	30.50	a — d	(78.0)	55.6
b	0.50	29.25	b — d	(63.2)	
c	0.70	26.50	c — e	55.0	
d	0.90	23.25	d — e	56.2	
e	1.50	19.00			

less than expected on the basis of the relatively constant non-solvent volume calculated from the data on more concentrated solutions. The cause of this apparent deviation from purely osmotic behavior is probably, by analogy with the erythrocyte, to be sought in loss of solutes into hypotonic media.

A quantitative study of swelling and shrinking of human and rabbit erythrocytes was made by SHAPIRO and PARPART (1937) using hypotonic and hypertonic solutions and making measurements by means of the photoelectric method. They found that the permeability constant (k) for human leucocytes for endosmosis was 1.35 and that for rabbit, 0.29. In the case of exosmosis, the permeability constant was about four times that for endosmosis.

CHAPTER VII

THE WATER RELATIONS OF ANIMAL TISSUES

Muscle. Vertebrate striate muscle will serve to illustrate the pitfalls attendant upon any effort to determine the permeability of tissue cells to water. The osmotic relations of muscle have been studied for many years, some of the earlier work such as that of RANKE (1865) being still valuable. But muscle has been the subject of a prolonged controversy between proponents of "imbibition" and "osmosis", which has not yet subsided. In Chapter II we have tried to make clear the fallacy of this supposed distinction, and we shall not discuss this literature in detail¹).

In general muscles separated from their normal circulation and immersed in salt or sugar solutions of graded osmotic pressures respond to osmotic forces in a normal direction at first, but fail to attain the expected equilibria of volume or weight, and after one or several hours change their direction or rate of volume change to something quite uncorrelated with the supposed osmotic gradients. All the claims that muscle water relations are controlled by imbibition have been based upon the latter type of change. Typical curves showing these successive stages are given for example by FISCHER (1908) and MEIGS (1910). MEIGS points out quite clearly that irritability is lost when the

¹) Among those who have stressed the importance of "imbition" by living muscle are FISCHER (1908), MEIGS (1912) for smooth muscle only, OSTWALD (1916), WESSBERGE (1918), GELLHORN (1925), and ERNST and CZIMBER (1931), as well as those who have believed that considerable amounts of water were bound in muscle, including RUBNER (1922), THOENES (1925), HURTHLE (1931), KEHAR and MCCOLLUM (1934). The dominant importance of osmosis in the then prevalent restricted sense has been urged by LOEB (1897) and his students COOKE (1898), WEBSTER (1902), BEUTNER (1912, 1913) and v. KÖROSY (1914) and by FLETCHER (1904), BUGLIA (1909), SCHWARZ (1911), MEIGS (1910, 1912), and MEIGS and ATWOOD (1916) for striate muscle, and later workers.

anomalous volume changes set in and regards the muscle as at that time losing its semi-permeability. This explanation is criticized by LAUGIER and BÉNARD (1911) because muscles which are just beginning to lose weight in a hypotonic NaCl solution will still swell if transferred to distilled water. LAUGIER and BÉNARD neglect that a muscle consists of many cells most of which may have lost their semi-permeability at a time when enough uninjured cells are still present to make the muscle swell in distilled water. The same reservation must be made with respect to the use of irritability as a criterion of absence of injury. In fact it is to be suspected that entire frog's gastrocnemii when merely immersed and not perfused quickly suffer death of some of the cells. HILL (1930) records an observation by HORTON to the effect that a frog gastrocnemius suspended in RINGER's solution may lose 25% of its potassium in 5—6 hours. The same muscle *perfused* with RINGER's solution for 5—6 hours loses only about 6% of its potassium (ERNST and SCHEFFER 1928).

Muscles immersed in any solution are, therefore, under such unfavorable conditions that after the first hour or so they cannot be regarded as uninjured nor can data taken after that time be regarded as having any significance for living muscles. Even before there is any serious effect on permeability there is probably an increase in the osmotic pressure of the fibres. HILL and KUPALOV (1930) showed that this was one effect of CO_2 , which is normally produced by resting muscle. This throws out a large part of the recorded data. The source of the changes probably lies in the accumulation of products of metabolism, but here our knowledge is not sure. It would be exceedingly valuable if data could be obtained as to the volume changes of muscles *perfused* with solutions of different osmotic pressures, or of isolated muscle fibre in similar solutions.

Another source of error in the earlier work was the inter-comparison of anisotonic solutions. LOEB (1897) for example compared 0.125 M NaCl with 0.100 M CaCl_2 , their freezing points being about -0.432° and -0.479° respectively. It is not surprising that muscles which barely gained weight in the former lost weight in the latter. The difference in behavior cannot be attributed to specific ionic effects.

Examining the data with the above reservations one finds that uninjured resting frog muscle during the first hour neither

absorbs nor loses appreciable amounts of water when immersed in 0.125 M NaCl. In anisotonic solutions it loses or gains water at rates which are very roughly proportional to the imposed activity gradients. The curves in Fig. 11 taken from BUGLIA's paper (1909) illustrate this point.

The driving forces for water transfer between muscle cells and the external medium seem to be nearly or quite exclusively activity or osmotic gradients. The muscle fibres which alone (and not the whole muscle) are the sites of semi-permeable plasma membranes (OVERTON 1902) should then individually swell or shrink to equilibrium volumes calculable from their initial osmotic pressure, that of the medium, the non-solvent volume, etc. From the whole muscle it would be necessary to add to the non-solvent

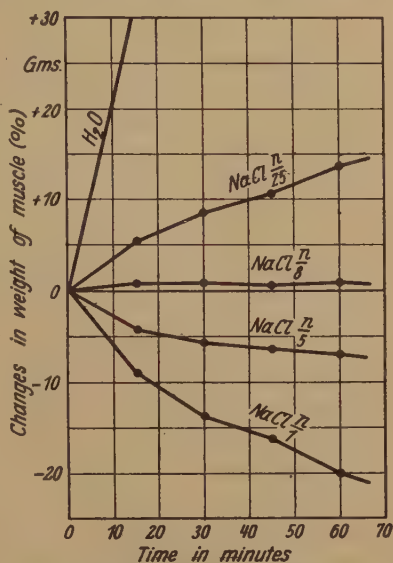


Figure 11. Relation between changes in weight of muscle and activity gradients.

volume a correction for intercellular spaces and connective tissue which would be largely unresponsive to osmotic forces. The magnitude of the intercellular or tissue spaces has been determined by FENN, COBB and MARSH (1934), determining the chloride content of whole frog skeletal muscles. They assume with apparent justification, that chloride is present only in the tissue spaces, and that the chloride concentration in the intercellular fluid equals that in the immersion fluid. The average tissue space was found to be about 15 per cent of the fresh muscle volume. The indirect conclusions of HILL (1930) give a similar result, viz: 13 per cent.

Unfortunately, equilibrium in the most used muscle, frog gastrocnemius, is not even approximately reached before injury sets in, even in the most favorable solutions. HILL (1930) has fitted three points of a weight-time curve for frog's gastrocnemii

to the theoretical time curve of swelling of a cylinder composed of semi-permeable fibres and deduced therefrom that even after more than seven hours swelling was only about 75 per cent complete. Although this conclusion is open to several objections (such as the possibility that the observed time-curve may have been warped by injury to a portion of the fibres) and therefore cannot be relied upon very exactly, still it is clear that equilibrium is not attainable in experiments as they have heretofore been conducted. This applies to all muscles so far studied, as well shown by FENN (1936) for several frog muscles. These muscles immersed in hypo- or hypertonic RINGER solution show with lapse of time increase in "chloride space", i. e., tissue space, progressive decrease in volume, and impairment of tension produced as well shown by FENN (1936) for several frog muscles. In hypotonic solution, temporary increase of the last two measures preceded the progressive decrease.

In view of errors due to faulty isotonicity of solutions, neglect of non-solvent volumes which in frogs' gastrocnemii amount to about 20.5% (FENN 1936), and failure to attain true equilibria it is not surprising that LOEB (1897) and so many subsequent workers failed to find the result which they expected, that is: a simple reciprocal relation between the supposed osmotic pressure of the medium and the weight of the muscles after long immersion. The non-appearance of this relation: $\Pi_0 V_0 = \Pi_1 V_1 = \Pi_2 V_2 = \dots$ led FISCHER (1908) and many others to the unjustified conclusion that osmotic forces in living muscle were negligible.

Since no experiment by present methods can yield equilibrium relations directly, HILL (1930) has attempted to arrive at such a result by appropriate control experiments which yield corrections for the presumed changes in the muscle and calculation therefrom.

For details see pp. 497 ff. of the original paper. From the calculated equilibrium volumes in slightly diluted or concentrated RINGER solutions, extrapolated to muscles of weight = 0 in order to minimize errors inherent in large masses of tissue, HILL found that the volume change corresponded to that expected in an osmometer whose weight was 0.47 times that of the muscle. Since HILL and KUPALOV (1930) had previously calculated that 0.77 times the weight of the muscle represented the amount of "free" water, — total water being 0.816 (KATZ 1896), 0.783

(ERNST and SCHEFFER 1928), or 0.795 (FENN 1936) — this result means that the volume changes were only about 61% as great as expected. HILL believes that this discrepancy is due to the fact that about 0.13 gm. out of the 0.78—0.82 gm. H_2O /gm. muscle lies between and not within the fibres (cf., FENN's (1936) tissue space of 15%), together with loss of semi-permeability by about 25% of the fibres. This conclusion appears reasonable, even though it cannot be regarded as conclusive proof of the purely osmotic nature of the water exchanges of these muscles.

ERNST and CZIMBER (1931) object to HILL and KUPALOV'S assumption, quoted above, that all of the crystalloids and all or nearly all of the water in fresh muscle are "free". But independent methods of investigation have confirmed, at least for muscle in rigor, the supposition that all or nearly of the water is free. (JENSEN and FISCHER 1910; JENSEN 1913; MENNIE 1932; BROOKS 1933, 1934a, 1934b.) Errors responsible for such divergent results as those of COLLIP (1920), RUBNER (1922), THOENES (1925) and KEHAR and MCCOLLUM (1934) are discussed by SALT and HINMAN (1932). ERNST and CZIMBER, using a wholly different method, believe that previously dried muscles bind some water. The method is not quantitative.

FENN (1936) has attempted to make direct measurements of the osmotic response of frog muscles, of which he had determined the normal tissue space (FENN, COBB and MARSH 1934). During the immersion of these muscles in hypo- and hypertonic NaCl solution, and increase in "chloride space" was observed. This may be considered to be a measure of the number of dead or dying muscle fibre cells. The volumes of fibres in these solutions indicate widely varying proportions of osmotically inactive water. However the mean, 15%, may well mean that a corresponding proportion of the fibres have died, thus agreeing with the increase in chloride space. This result confirms the present comparative absence of positive proof of osmotic changes of striate muscle fibres. The solitary significant bit of evidence is that due to BUGLIA (1909).

The effects of various factors on the driving forces for water in muscle are for similar reasons difficult to evaluate. It appears certain that muscular activity increases the osmotic pressure within the muscle fibres. (RANKE 1865, COOKE 1898, FLETCHER 1904, SCHWARZ 1911, MOORE 1916, MEYERHOF 1930,

HILL and KUPALOV 1930, HILL and PARKINSON 1931.) The increase may correspond in the case of completely fatigued frog sartorius muscles to the increase from 0.726 to 1.076% NaCl, is not a result of lactic acid production exclusively and does not seem to be explicable in terms of reactions known to take place during muscular contraction (HILL and PARKINSON 1931) unless we assume that bound salts are freed as supposed by ERNST and SCHEFFER (1928). FENN and COBB (1936) show that rat muscle stimulated through the nerve gains intracellular water as well as extracellular water (containing sodium chloride presumably at isotonic concentrations). The changes are all largely reversible during one to 3 hours of recovery.

PONDER and GAUNT (1934) find the muscles of adrenalectomized rats to swell more slowly in diluted RINGER-LOCKE's solution than those of normal rats. PUDDU and VALLOGINI (1934) report effects of hormones on the swelling-time curves of mammalian muscle. These may both involve permeability as well as, or instead of, driving force.

The effects of ions on the swelling of striated muscle have been studied by LOEB (1897), WEBSTER (1902), OVERTON (1902), FISCHER (1908), v. KÖROSY (1914), GELLHORN (1925), BRUGSCH, CAHEN, HORSTERS and ROTHMANN (1927), VIOLLE and DUFOURT (1928), VANDERVAEL (1934) and others. For reasons discussed above most of these studies may be neglected. Thus, GELLHORN (1925) and BRUGSCH, CAHEN, HORSTERS and ROTHMANN (1927) observed effects upon injured or dead muscles. VIOLLE and DUFOURT (1925) compared "equally concentrated" solutions but do not state whether they refer to equal percentage, equal molarity, equal normality, isotonicity or what. How is it possible to interpret such experiments? There do appear to be characteristic effects of the ratio of Ca or Sr to alkali metal ions on the later stages of the volume-time curves. These probably depend upon the relative toxicity of the solutions, some of which injure the muscle fibres sooner than do others. Their data as to the relative "hydrating powers" of various ions may well be connected with the relative penetrability of these ions complicated by differences in the initial osmotic pressures of the media.

The experiments of VANDERVAEL (1934) on brain and muscle tissue of mammals show a similar disregard of the need for isotonicity of the media compared. When, for example, VANDER-

VAEL adds 0.02 N CaCl_2 to isotonic glucose the resulting shrinkage is probably not a specific ion effect but the result of an increase of osmotic pressure of nearly 20%! The author claims that muscle, like brain tissue, which he also studied, loses weight when 5% isoelectric gelatin is added to otherwise isotonic glucose solution. His published figures contradict this assertion. The gelatin would have an osmotic pressure of the same order of magnitude as the colloid osmotic pressure of muscle which DUFF (1932) by direct measurement found to be 0.049 to 0.063 atm. If it became somewhat ionized it might also approximately compensate for the membrane equilibrium effect of the muscle proteins. The action of colloids in preventing oedema of perfused frog head — foreleg — heart preparations was recognized and interpreted in the light of such considerations by GAYDA (1916, 1927).

It has also been recognized since the early work of LOEB (1896) that acids and bases affected the swelling of muscle. It is impossible to determine the relative importance of (1) the direct penetration of the added acids or bases, (2) their effect on the dissociation of proteins and other weak electrolytes within the muscle fibre, and (3) their effect on permeability. Some of these experiments will be referred to in discussing the last of these.

Rate. The initial rates of swelling of isolated frog gastrocnemius and upper-leg muscles were found by ADOLPH (1931) to be proportional to the square root of the time elapsed since the immersion of the tissue in the medium, and of the same order of magnitude as in the case of frog skin, normal intact frog and isolated skin, but showing distinct differences. More recently, PARRY (1936) concluded that swelling depends upon the surface area of muscle tissue exposed to the medium, and that the rate depends upon the osmotic pressure of the medium, both colloid and crystalloid.

The osmotic properties of smooth muscle

MEIGS (1912) compared the swelling and shrinkage of smooth muscle from the stomach of the frog with that of striated muscles of the same animal, and was thereby led to conclude that the behavior of smooth muscle was essentially non-osmotic. GELLHORN (1925) found the same smooth muscles to be like the striated muscles in their response to certain special sequences of prolonged treatment with very anisotonic salt solutions. We believe the

striated muscles to have been dead or at least in part seriously injured in these experiments of GELLHORN's, and that as a consequence neither the striated nor the smooth muscle exhibited "ideal osmotic" behavior. There is thus no disagreement with MEIGS. SERENI's experiments with *Octopus* chromatophores, which are under the control of smooth muscles, seem to lead to similar conclusions (SERENI 1927). It might be suspected that smooth muscles are so very susceptible to injury as always to have been injured by the experimental procedure. If this be not true, then it seems necessary to suppose that smooth muscle fibres are so permeable that their behavior is dominated by the swelling and shrinkage of cell colloids. Although this is in itself osmotic (See Chapter II) yet it makes it impossible to calculate the osmotic gradients, i. e., the activity gradients of water, on the basis of the information available.

We shall, therefore, attempt no estimate of the absolute permeability of smooth muscles to water

The absolute permeability of striated muscle fibres to water

It is impossible to make a satisfactory quantitative calculation of the absolute permeability of striated muscle fibres to water. A rough estimate may, however, be attempted. The difficulties to be met are (a) the complex structure of the individual muscle fibres, the surfaces of which are presumed to be the semi-permeable membranes, and (b) the fact that these semi-permeable fibres, together with their binding and connecting tissues, the sarcolemmas, endomysia, etc., are built up into a more or less solid mass, so that none except the outermost layers may be in immediate contact with the experimental solution¹). In regard to the internal complexity of the striate muscle fibres it may be surmised that it is not a controlling factor in the rate of water exchanges by the fibre, the latter being controlled by the permeability of the plasma membrane of the fibre in the same way that the slowest of a series of consecutive reactions becomes the "master reaction", which determines the reaction rate of the whole chain. If this be assumed, then the rate of water exchange

¹) For further details about muscle structure and its significance see MAXIMOW and BLOOM (1934) and MEIGS (1932).

by muscle fibres will depend upon the driving force across them, and in the absence of any proof that other than osmotic forces act, we shall confine our attention to them.

But when a whole muscle is plunged into a hypotonic solution, how soon do the interior fibres feel the full osmotic gradient? Following OVERTON (1902) we may consider the sarcolemmas, epimysia, etc., to be so permeable as to play no controlling rôle. Yet solutes must diffuse out of intercellular spaces (or water in) relatively fast, otherwise osmotic gradients will be set up first across the outer faces of the outermost fibres which will gradually admit water diluting the sarcoplasm of these fibres, thus setting up gradients across the inner surfaces of these fibres, then the outer surfaces of the next layer, and so on. If this occurred, swelling of the inner fibres would follow a sigmoid time curve like that of the second and following reactions of a series of consecutive unimolecular reactions¹), and the swelling of the whole muscle should begin slowly, accelerate, and then slow down as equilibrium was approached. In the most favorable cases, where rather small muscles, such as the sartorii of small frogs, are used, such an initial lag has not appeared when readings were taken at five minute intervals. It may be estimated that the whole thickness of this muscle comprises only 10 or 15 fibres, and that in this case the intercellular fluids so quickly reach equilibrium with the immersion fluid that the osmotic gradients for all the fibres may be taken, as a first approximation, as being equal, and the same as that to which the outermost fibres are exposed.

We may, therefore, assign to individual striated muscle fibres approximately the permeability to water which can be calculated from the relative increase or decrease in weight shown by frog sartorius muscles in hypo- or hypertonic solutions. Somewhat better data are available for frog gastrocnemius muscles (BUGLIA 1909, v. KÖROSY 1914); but their larger size makes the neglect of intercellular diffusion processes less justifiable.

MEIGS (1910) gives the best available data, according to which a frog sartorius muscle originally weighing 150 mg. weighed 215 mg. after five minutes' immersion in distilled water. (Data taken graphically from MEIGS' Fig. 2). This is a gain of approximately 44%. Assuming that each muscle fibre gained in this proportion and considering the fibres to have been cylindrical

with a diameter of $100\ \mu$, we find that each linear cm. of muscle fibre has a surface of $0.01\ \pi\ \text{cm.}^2$ and takes in $0.061 \times 10^{-5}\ \pi\ \text{GM.}$ of water in the first 300 seconds with an initial activity gradient of water (equivalent to the osmotic pressure of 0.726% NaCl) of about $0.25\ \text{GM.} \times \text{liter}^{-1}$. Making no correction for change of surface area or gradient, and calculating the mean value for the whole period, we obtain a permeability.

$$P = \frac{0.061\ \pi \times 10^{-5}\ \text{GM}}{0.01\ \pi\ \text{cm}^2 \times 300\ \text{secs} \times 0.25\ \text{GM} \times \text{liter}^{-1}} \\ = 8.1 \times 10^{-7}\ \text{GM} \times \text{cm}^{-2} \times \text{sec}^{-1}\ (\text{GM} \times \text{liter}^{-1})^{-1}.$$

This value is of same order of magnitude as that found for the cells previously discussed. Considering that it is an exceedingly rough approximation this result is interesting.

Smooth Muscle Cells. MEIGS (1932) gives several reasons for supposing the smooth muscle fibres to have no semi-permeable plasma membranes. These include swelling or shrinkage in salt solutions (which depends upon the nature of the ions present rather than upon the osmotic pressure), the lack of any marked irreversible change in this behavior upon subjecting them to conditions which appear to destroy the semi-permeability of striated muscle, and the failure of the fibres to go into rigor when cut across transversely as do the fibres of striated muscles under similar conditions. This was shown for invertebrate smooth muscle (adductor of *Venus mercenaria*) as well as for that from the gut of the frog (MEIGS 1914). Unless there is some undetected error it seems necessary to conclude that smooth muscle fibres lack any well-developed semi-permeable plasma membrane, and are, therefore, easily permeable to many solutes and to water.

Nerves. Many experiments have been done on the swelling of nervous tissues, especially brain and spinal cord, by prolonged immersion of large pieces in different solutions (BAUER 1911; BALDI 1932). This same error may have affected also the experiments of NETTER (1927) on sciatic nerves of frogs whose volumes he measured in diluted and hypertonic RINGER's solutions. From rather inadequate data he concludes that the non-solvent volume of such a nerve is about 40% but that when the osmotic pressure is less than about half that of an isotonic solution the "membranes"

¹⁾ See, for example, MELLOR 1926.

of the nerve restrict its swelling. His calculations are marred by errors, and it can only be supposed that if there were a non-solvent volume of between 40 and 60% the data would accord with purely osmotic water relations (except in markedly hypotonic solutions). The water content of the whole sciatic nerve of frogs is g as 74.6% (GERARD 1932). If we suppose that 30% of the nerve is intercellular material (as NETTER supposes), and that most of this is water, then NETTER's non-solvent volume of 40—60% becomes reasonable. This evidence is not very satisfying.

REYNAULD (1908) perfused dogs' brains with hypo-, iso- and hypertonic saline solutions and found them to swell, remain constant, and shrink respectively, as would be expected on the basis of the osmotic relations. REYNAULD points out that abnormal osmotic pressures rapidly injure the brain cells and cause them to lose their semi-permeable character.

Altogether these experiments suggest that the water intake and outgo of tissues is controlled primarily by osmotic pressure differences. No experiments have been done which would allow calculation of their absolute permeability, and the operation of other than osmotic driving forces is by no means disproven. Furthermore, the rôle of the nerve sheath in permeability of nerves to water has never been studied. Quite possibly it forms a relatively impermeable sheath, so that water must get into the axone through the nodes of RANVIER. The lipoids in nerve sheaths, occurring in repeated double layers (See review by SCHMITT 1939), must constitute a relatively water-impermeable layer.

FENN (1937) shows that only doubtful increases of water content of cat nerves follow stimulation. It is quite possible that changes in driving forces occur, but obviously small, during nerve activity.

Osmosis into frog skin, and exchanges of water in the frog have been studied extensively and quantitatively by ADOLPH (1931, 1933). The initial exchanges of water by the intact frog is referred to that rate of intake by frog skin. This was found to be about $36 \text{ cm.}^3 \text{ per cm.}^2 \times 10^3 \text{ per minute}$. The circulation has no influence upon the rate of osmosis in or out, whereas the nervous system does (ADOLPH 1934), [See also RUBINSTEIN (1935)], whereas McCLENDON (1914) states that water passes through the frog skin on an osmotic basis only. In the case of

frogs from which the skin had been removed, the initial rates of water uptake were proportional to the concentrations of NaCl in the medium (ADOLPH 1931). For a review of water exchanges in the frog see ADOLPH (1933).

BATEMAN (1934) states that the gill membrane of the crab is almost impermeable to water, and that the body fluids exposed to various external conditions show that the crab has temporary osmotic independence. During the molt cycle BAUMBERGER and OLMSTED (1928) found changes in the osmotic pressure and water content of crabs. In the case of medusae, BATEMAN (1932) found that there is no osmotic regulation and that the activity of the water is the same as that in sea water. See also BETHE (1934) on the water permeability of the body surfaces of various aquatic animals. Experiments were made on the body weights and the freezing point depressions of the body fluids of three crabs and a pulmonate mollusk of the Australian mangrove swamps by DAKIN and EDMONDS (1931). They concluded that osmotic readjustment was due to the movement of solutes rather than water.

SMITH, H. W., (1930, 1931) states that there is a selective sorting out of the ions of sea water by different organs of the fish. They swallow sea water, absorb Na, K and Cl, while Mg and Ca remain in the intestine. There is a constant water content and a constant salt content of body fluids. When eels are transferred from fresh water to sea water, the serum proteins decrease about 40%. Conger eels show no such changes (BOUCHER-FIRLY 1934).

CHAPTER VIII

OSMOTIC RELATIONS OF PLANTS

Schizophyta. Bacteria. Among the papers dealing with osmotic phenomena in bacteria the only ones meriting any attention in connection with permeability are those of FISCHER (1891, 1903). In spite of difficulties of observation and the necessity of making the final studies in fixed and stained preparations, there seems to be no cogent reason for doubting that he observed plasmolysis of many species of bacteria. He studied in particular detail the behavior of *Vibrio comma* SCHROTER. (*V. cholerae* of auths.)¹⁾ Unstained living cells of this species could be seen to undergo characteristic apparent fragmentation into refractile granules whenever corresponding fixed and stained material showed plasmolysis. Plasmolysis was always characterized by the appearance of convexly bounded clear spaces in the protoplasm which seldom withdrew from the ends of the cells. The culture media had an osmotic pressure equivalent to that of 0.04 M NaCl, and plasmolysis was found whenever the bacteria were transferred to NaCl solutions of 0.08 to 0.10 M or above. Maximum plasmolysis was almost instantaneous and in 0.2 M NaCl recovery occurred in 1 to 2 hours or less. The following bacteria exhibited similar behavior: *Vibrio proteus*, *V. saprophiles*, *Spirillum undula* and other species, *Eberthella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *P. fluorescens*, *Serratia marcescens*, *Micrococcus can-dicans* and unspecified species of *Crenothrix* and *Sphaerotilus*. Other authors have reported similar phenomena in *Spirillum giganteum* except as the species is not there referred to. In that case the name is that given in the paper quoted. (= *Rhodospirillum giganteum* MOLISCH. ?) (ELLIS 1903, VAHLE 1909; and possibly SWELLENGREBEL 1907) *Vibrio proteus* (GARBOWSKY 1907) in

¹⁾ The nomenclature here used is that of BERGEY (1930).

0.5% NaCl but not in 0.1%; while VAHLE (1909), HOLLING (1911) and RAICHEL (1928) have confirmed FISCHER's results for various species. RAICHEL used ultramicroscopic observation, and found the spaces in the cytoplasm of living "plasmolysed" cells to be optically empty. This strengthens the evidence that true plasmolysis actually occurs. Many workers supposed to have observed plasmolysis have made preparations under such condition, that it is, to say the least, uncertain that plasmolysis of living cells occurred (TRENKMANN 1890, NOVY and KNAPP 1906, SWELLENGREBEL 1907, 1909, SCHUSTER 1910), while uncertainty as to the nature and cause of plasmolysis, which has by some been regarded as osmotic swelling, make it valueless as a criterion of permeability to water (FISCHER 1900, 1906, LEUCHS 1905, GARBOWSKY 1907, RAICHEL 1928).

From the evidence thus presented it is clear that the bacteria listed above are all permeable to water. The degree of permeability may not be as great as the almost instantaneous plasmolysis suggests since the specific surface (ratio of surface to volume) of such exceedingly small cells is very high. Even supposing an amount of water equal to half the cell's volume to be withdrawn from a cell about $1 \times 5 \mu$ in 5 seconds by an osmotic difference equivalent to 0.1 M NaCl such as FISCHER used, we still find the permeability to be only about 6.5×10^{-1} GM. $\text{cm}^{-2} \cdot \text{sec}^{-1} \cdot (\text{GM. l}^{-1})^{-1}$ which is of the same order of magnitude as the permeability to water found in other forms by more exact methods.

Many types of bacteria have been observed to be not plasmolysed under any conditions. FISCHER (1903) lists the following: *Bacillus anthracis*, *B. subtilis*, *B. megatherium*, *B. mesentericus*, "*B. peptonificans lactis*", *B. lactis acidii*, *Proteus vulgaris*, *Sarcina* spp., *Staphylococcus* spp., *Mycobacterium tuberculosis*, and *Corynebacterium diphtheriae*. These he regarded as being so permeable to the solutes used that the osmotic gradients set up were too transient to effect plasmolysis. Of course his experiments do not exclude complete impermeability to water as the cause, but with the possible exception of acid-fast forms with waxy envelopes, such an explanation is improbable. They are probably as permeable to water as for the cells which could be plasmolyzed.

Other bacteria which could not be plasmolyzed included *Beggiatoa mirabilis* (HINZE 1902, RUHLAND and HOFFMANN

1924). Various spirochaetes such as *Borrelia gallinarum* (PROWAZEK 1906), *Cristispira balbianii*, and *C. anodontae* (HOLLING 1911), and certain Myxobacteriales (*Myxococcus ruber*, *Polyangium fuscum*, and *Chondromyces crocatus*) (VAHLE 1909). Since at least some of these appear to shrink in hypertonic solutions (*Cristispira* spp. and *Borrelia* spp.), it seems probable that they are permeable to water, but that their membranes or cell walls if any, are elastic and shrink as much as the protoplasm does.

We may therefore conclude that bacteria in general have roughly the same permeability to water as other living cells.

Schizophyta. Algae. The osmotic responses of a brine organism, *Microcoleus chthonoplastes* THUR., are discussed by CAVARA (1902). This blue-green alga finds its optimum environment in sea water, whose osmotic pressure is 23.8 atm. In distilled water most of the cells of the filament take in water and burst, while in relatively concentrated brines, whose osmotic pressure is about 150 atm.¹⁾, an irregular plasmolysis occurs. In solutions of other salts, slightly higher concentrations are necessary for plasmolysis. If the alga is allowed to reach this stage slowly, this plasmolysis may be smooth and spherical, a condition which may depend on the prolonged absence of calcium. Even in salt heaps the alga survives, and on return to sea water, it returns to active life.

These phenomena show that these cells are permeable to water in response to osmotic driving forces, and that this water transfer is reversible. Although these facts are interesting, no quantitative conclusions can be obtained.

RESÜHR (1935), taking into account the non-solvent volume in the case of unfertilized *Fucus* eggs, found by a modification of the BOYLE-MARIOTTE-VAN'T HOFF law and FICK's diffusion equation, that k' , which he designates as the "Wasserpermeationskonstante", = 0.0253 (μ). See also the work of KOTTE (1915) and BÜNNING (1934) on marine algae.

Fungi. There are several papers which discuss the water relations of filamentous fungi, notably those of ESCHENHAGEN (1889) (which we have not seen), MAYENBERG (1901), and PANTANELLI (1904). VAHLE (1909) also mentions experiments on the plasmolysis of fungi. *Aspergillus niger* has usually been used, and

¹⁾ This corresponds to the brine as it leaves the pickle pools, having lost its calcium salts, and in which NaCl is approaching saturation.

interest has been centered largely upon the means by which fungi maintained their turgor in culture solutions of high osmotic pressure. MAYENBERG (1901) showed by analysis of extracts that when the principal solute in the medium was glucose or any of several common inorganic salts, the solute in question was not present in the cell sap in significant amounts. The turgor of the hyphae was therefore due to the effect of other solutes; by elimination MAYENBERG was led to suspect the presence of unstable metabolites. PANTANELLI (1904) postulates that the total water attracting power of the cell contents is made up almost entirely of the osmotic pressure of the vacuolar sap *plus* the swelling pressure of the protoplasm, the magnitude of each of these pressures being proportional to the volume of the corresponding phase. The absurdity of this concept is obvious.

It is not necessary to assume either hypothetical solutes or special water motive forces to account for the high osmotic pressures necessary to plasmolyse fungus hyphae grown in concentrated solutions of non-penetrating solutes. Neither need we postulate mysterious "swelling pressures" as PANTANELLI does. As has been explained, the activity deficit of water in concentrated solutions is sufficient to account for the phenomena on an osmotic basis. MAYENBERG's determinations of the water content of mats of *Aspergillus* hyphae indicate a rather dilute protoplasm, but are probably vitiated by the inclusion of interhyphal water and of much dead material (PANTANELLI 1904). In all probability the protoplasm of living fungus filaments becomes very concentrated when the outside medium has a high osmotic pressure. On the other hand, it must be emphasized that our present knowledge does not exclude the possibility that unknown solutes are produced by the cells as a means of osmotic regulation as MAYENBERG (1901) suggests, or that undetected water motive forces may operate. The former process which v. RYSELBERGHE (1898) has termed anatonosis appears to occur in vascular plants and is discussed below.

The interest of all of the workers being centered largely upon osmotic regulation over long periods, they have published no data on the *rates* of plasmolysis. For this reason no estimate at all can be made as to the absolute permeability of these fungi to water.

Fungi; yeasts. But a certain amount of work has been done with the ellipsoid cells of yeasts, notably with different

strains of *Saccharomyces cerevisiae*. The experiments of PAINE (1911) dealt with yeast cells taken from pressed cakes, and these cells were of doubtful normality (BEETLESTONE 1928). But the later work of BEETLESTONE (1930) confirms PAINE's conclusion that water equilibrium in non-electrolyte solutions is attained after several hours; more detailed study of the swelling of yeast cells in distilled water and the shrinkage in 10% sucrose (Figure 12) shows that substantial equilibrium is attained in 3–4 hours.

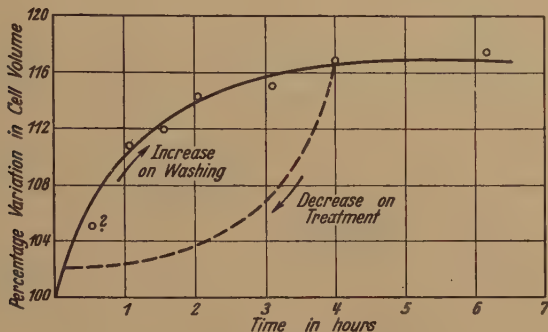


Figure 12. Percentage variation in cell volume of yeast as influenced by treatment of cells.

The exponential form of these curves is well shown, and from this, together with other data given, it should be possible to calculate the permeability. The volume of these cells in distilled water was found to be about 2×10^{-10} cm.³ and the cell was a short prolate ellipsoid having a surface not far from an equally large sphere, therefore about 1.65×10^{-6} cm.² The value of b , the non-solvent volume, when calculated from volumetric data is so inconstant as to arouse suspicion (BEETLESTONE 1930, Table II). But the analytically derived value, $b = 46\%$ (BEETLESTONE 1928), is more satisfactory. This depends on the ratio of dry solids to water. Calculating from the shrinkage in 10% sucrose, where the osmotic forces are known quantitatively, and using LEITCH's Equation 7a (1936), we obtain the result that $P = 0.415 \times 10^{-7}$ GM · sec⁻² (GM · liter⁻¹)⁻¹. This result is low but of the expected order, although the assumptions may well be subject to at least a twofold uncertainty. There is no evidence as to possible electrical forces, and unnatural deductions might be

drawn as to the permeability of yeast cells to carbohydrates. This is discussed in Chapter XI.

Vascular plants: A great deal of work on osmotic exchanges in vascular plants has been done. These results deal mainly with degrees of plasmolysis and deplasmolysis of the cells. Much of this work cannot be evaluated quantitatively. A few of the more recent experiments, however, are given below.

LEVITT, SCARTH, and GIBBS (1936) have taken into consideration the decreasing thickness of the protoplasm layer with dilation of the cell as water is taken in. Using the isolated protoplasts of onion pulp they found by photomicrographic measurements of changes in volume with increased swelling and deplasmolysis that the normal rate of penetration of water into cells is about $20\ \mu$ per hour at 20°C . at one atmosphere pressure. There was a gradual and relatively steady rise to a little beyond normal size of the cell and then a sharp jump upward caused by the mechanical effect of the stretching membrane.

HUBER and HÖFLER (1930) have measured the permeability of a large number of cells to water. These measurements include a large variety of plants, not only algae but also cells of vascular plants. By means of plasmolysis and deplasmolysis experiments with various salts and sugars, they deduced that in the case of *Salvinia*, the rate of penetration is (according to a modification of FICK's law) $33\ \mu$ per hour — a small rate, as compared, for example, with that of *Majanthemum* which was 120 to 240 times as high as that for urea to 10,000 as high as that for glucose. They conclude that the rate of penetration is determined, not by the plasma membrane, but by the protoplasm itself.

ERNEST (1935) believes that the plasmolytic method is inapplicable in the estimation of osmotic values of plant cells, because when mechanical injury and the pathological action of fluids from ruptured cells are eliminated, the limiting plasmolysis so much deferred that secondary changes have taken place in the cells.

ILJIN (1935) also attributes change of the turgor of plant cells as the cause of their death. Mechanical stretching and change in turgor was noted by CORRENS (1891) when plasmolysis occurred in the connecting hairs of blossoms of *Aristolochia*.

DE HAAN (1933) regards the whole protoplasmic layer as a homologous membrane whose permeability is positively correlated with its degree of swelling.

ALBACH (1931) finds that the osmotic pressure of younger cells of *Rhoeo discolor* is much greater than that of older cells.

BAPTISTE (1935) found that the effects of certain cations on the permeability of discs of potato and carrot were in the descending order: K, NH_4 , Na, control, Mg, Ca.

KAHO (1935) shows that the influence of alkali salts on the deplasmolysis of young epidermal cells of *Allium cepa* does not obey the lyotropic series for cations and anions.

The work of COLLANDER (1934) who worked on *Rhoeo discolor* is discussed under non-electrolytes.

STILES and JORGENSEN (1917) studied the swelling of carrot and potato discs and concluded that the rate is not exponential and differs for the two tissues.

URSPRUNG (1926) has used the root tips of *Vicia faba* and has tried to deduce quantitative results by means of the formula

$$S_z = S_j - W$$

in which S_z is the suction force of the cell; S_j , the suction force of the protoplasmic contents of the cell; and W , the wall pressure. Quantitatively, his figures are wholly inapplicable to unvacuolated cells but probably apply loosely to vacuolated ones. It is also questionable whether one should use the concept "Saugkraft" rather than that of fugacity of water, as has been used throughout this monograph.

COOPER and PASHA (1935) noted that the osmotic and suction pressure of some species of mangrove vegetation was highest in the leaves, medium in the stem and lowest in the roots, and that there was a change with change in seasons.

Table XIV has been compiled from some of the available data in order to unify results. It is hoped that future experiments will record data in equivalent or similar form so that better comparisons can be made.

Table XIV. Permeability to water of various membranes. The figures of surface, per second, for a difference of 1 M in

Organism	Diameter (mm)	Tissue or cells	Shape	Direction of flow
Celloidin	—	—	—	—
"	—	—	—	—
"	—	—	—	—
Rabbit	—	Red blood cells	Disc	in
"	—	" " "	"	"
"	—	" " "	"	"
Sheep00478	Red blood cells	Disc	in
"00478	" " "	"	"
"00478	" " "	"	"
Arbacia074	Egg, unfertilized	Spherical	in
"	—	" fertilized	"	"
"	—	" artif. act'd	"	"
Arbacia0714	Egg, unfertilized	Spherical	in
"	—	" "	"	"
"	—	" "	"	"
Strongylocentrotus purpuratus0775	Egg	Spherical	in
Dendraster124	Egg, unfertilized	Spherical	in
"121	" "	"	"
"122	" "	"	"
Dendraster122	Egg, unfertilized;	Spherical	in
"124	jelly removed by	"	"
"121	NaCl and shaking	"	"
Patiria165	Egg	Spherical	in
"161	"	"	"
Pisaster1717	Egg	Spherical	in
Urechis caupo120	Egg	Spherical	in
" "120	"	"	"
" "120	"	"	"

Given indicate moles of water passing into or out of the cell per 1 cm² the activity of water inside and outside the cell.

K	External solution	t °C	P = Permeability × 10 ⁻⁷	Observer
—	—	—	0.055	NORTHROP (1929)
—	—	—	0.102	COLLANDER (1926)
—	—	—	.055—0.55	FUJITA (1926)
—	NaCl 0.096 M	—	0.0583	BROOKS (Original)
—	„ 0.144 M	—	1.94	„ „
—	„ 0.126 M	—	0.694	„ „
—	NaCl 0.126 M	—	0.69	PONDER (1934)
—	„ 0.096 M	—	5.86	„ „
—	„ 0.114 M	—	1.95	„ „
.1033	40% s. w. }	—	1.66	LILLIE (1916)
.416	60% tap. w. }	—	5.83	„ „
.29	—	—	4.44	„ „
.036	40% s. w.	24.8	1.75	MCCUTCHEON & LUCKÉ (1928)
.024	40% s. w.	20.5	1.25	„ „
.048	60% s. w.	24.8	1.13	„ „
.209	40% s. w.	16.4	3.13	LEITCH (1934)
.127	40% s. w.	20 ±	3.69	LEITCH (1931)
.141	50% s. w.	„	4.19	„ „
.164	60% s. w.	„	3.13	„ „
.128	40% s. w.	20 ±	3.63	LEITCH (1931)
.134	50% s. w.	„	2.83	„ „
.164	60% s. w.	„	2.66	„ „
.127	50% s. w.	20 ±	4.72	LEITCH (1931)
.124	60% s. w.	„	3.19	„ „
.115	50% s. w.	20 ±	3.11	LEITCH (1934, 1936)
.212	40% s. w.	20 ±	3.80	LEITCH (1934, 1936)
.219	50% s. w.	„	3.58	„ „ „
.209	60% s. w.	„	3.47	„ „ „

Organism	Diameter (mm)	Tissue or cells	Shape	Direction of flow
Rana sp.1	Striated muscle (sartorius)	Cylindrical	in
" "	"		—	"
" "	"		—	out
Spirogyra sp.03 × .1	Whole thallus	—	out
Tradescantia sp.	—	Lower epidermis of leaf	Hexagonal prism	out
" "	—	"	—	in
" "	—	"	—	out
" "	—	"	—	in
Tradescantia elongata .	.0142	Parenchyma cells	Spherical	out
Sambucus sp.	—	Pith	—	out
" "	—	"	—	in
Vicia faba	—	Lateral roots of seedlings	—	out
Taraxacum officinale .	.09 × .150	Lower side of leaf midrib	Hexagonal prism	out
" "	—	scape	—	out
Allium cepa	—	leaf	Hexagonal prism	out
Solanum	0.3	Large cells	Square	in
Daucus	0.3	Large cells	Square	in
Lamium purpureum .	.02 × .066	Epidermal cells	Cylindrical	out
" "02 × .068	" "	"	"

K	External solution	t °C	P = Permeability × 10 ⁻⁷	Observer
—	Dist. water	20 ±	1.11	MEIGS (1912)
.023	50% Ringer	18 ±	0.197	" "
—	20% " "	20 ±	0.388	" "
.086	KNO ₃ 0.2 M	20 ±	6.50	VAN RYSELBERGHE (1899, 1901, 1902)
.117	KNO ₃ 0.2 M	20 ±	6.94	VAN RYSELBERGHE (1899, 1901, 1902)
.140	" " "	"	1.94	"
.175	{ Sucrose isotonic with KNO ₃ 0.2 M	"	10.00	"
.150		"	2.05	"
.24	KNO ₃ 0.25 M	—	2.64	HÖFLER (1918)
—	Sucrose 0.731 M	20 ±	3.30	VAN RYSELBERGHE (1902)
—			0.278	
—	KNO ₃ 0.5 M	—	5.46	LUNDEGÅRDH (1911)
—	NaCl 0.22 M	25	3.22	BROOKS (1916)
.063	Sucrose 0.30 M	25	0.472	DELFT (1916)
—	Sucrose 0.18 M	20	1.66	DELFT (1916)
.003	Sucrose 0.26 M	—	1.11	STILES & JORGENSEN (1917)
.010	Sucrose 0.26 M	—	1.11	STILES & JORGENSEN (1917)
.458	KCl 0.5 M	22	2.97	WEIXL-HOFFMANN (1930)
.487	KCl 0.5 M	22	0.69	PONDER (1934)

CHAPTER IX

PERMEABILITY TO NON-ELECTROLYTES

The permeability of cells to non-electrolytes is largely of interest because there are no complications due to ionization of penetrating substances in the study of the nature of the plasma membrane.

The classification of this group is largely arbitrary and usually includes those bases and acids and their mutual salts whose k 's fall short of being 1% dissociated in the physiological pH range from about pH 4.0 to 10.0. In other words, those acids with a $k_a = 10^{-12}$ and those bases with a $k_b = 10^{-12}$ are usually classified as non-electrolytes. The following table gives the dissociation constants of some of those most generally used:

Table XV

Bases	k_b	Acids	k_a
Thiourea	1.1×10^{-15}	Glycerine	7.0×10^{-15}
<u>Urea</u>	1.5×10^{-14}	<u>Sucrose</u>	2.4×10^{-13}
Acetamide	3.1×10^{-15}	<u>Glucose</u>	6.0×10^{-13}
Alanine	5.1×10^{-12}	Phenol	5.8×10^{-11} or 1.3×10^{-10}
		H_3BO_3	1.7×10^{-9}
		Uric acid	1.5×10^{-6} (often included)
		<u>Ethylene</u>	6.0×10^{-15}
		<u>glycol.</u>	

Equilibrium

The question arises as to what is the driving force in the case of non-electrolytes? Does equilibrium depend upon the relative activities of the outside as compared with the inside

solution? This has been answered in the affirmative by one group of investigators in quantitative experiments, in which permeability has the dimensions, $\text{GM} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1} (\text{GM} \cdot \text{l}^{-1})^{-1}$, particularly in the case of the vacuolated cells, but also when non-vacuolated cells and epithelia were used.

BÄRLUND with COLLANDER (1929) showed by time curves of critical plasmolysis in the vacuolated cells of *Chara ceratophylla* that equilibrium is approached when $\Pi_1 - \Pi_0 = 0$. They used glycerol, urea, thiourea and lactamid. WILBRANDT (1931) showed the same results for *Rhoeo*, *Begonia* and *Basella* using additional compounds. These are the clearest quantitative cases for normal non-specialized cells.

The method of plasmolysis has been used by HUBER and HÖFLER (1930) in an extensive treatise on the permeability to water of *Majanthemum*. By measuring the size of the cell as it has pulled away from the walls, a quantitative value of degree of plasmolysis can be obtained. The same method has been used by ZEHETNER (1934) in a study of the relative plasmolysis of the cells of *Spirogyra* and *Hydrodictyon* in solution of the alcohols; by HÖFLER and STIEGLER (1929) to show the penetration of urea into *Gentiana sturmiana*; by HÖFLER (1934) in the case of the penetration of KNO_3 into *Allium cepa*; and by ILJIN (1935) to show the effects of salt and changes in pH in a number of vascular plants; by WEBER (1932) to show the effects of cold temperature.

Substances. PAINE (1911), using yeast, found that the ratio of EtOH inside the cell to that outside was about 85%, and for urea, 89%. The remaining 10 or 15% were attributed to the non-solvent volume. LUNDEGÅRDH and HOLBOLL (1926) found a ratio of 0.7 between the concentration of methyl acetate, fructose, galactose and urea inside and outside of red blood cells, while EGE (1927) attributes the discrepancy in the ratio of the equilibrium in red blood cells inside and outside to a 30% non-solvent volume.

On the other hand, other experiments show that equilibrium does not depend upon equal activity of the inside and outside of the cell, and that therefore there are other factors besides fugacity which are involved.

A great many isolated experiments have been done in which one or several non-electrolytes were used. WEBER (1930) found that old *Spirogyra* cells were more permeable to urea than young

ones. This is in keeping with the theory that complete permeability occurs when the cell is dead. SCHEITTERER and WEBER (1930) noted that the guard cells of *Ranunculus ficaria* and *Vicia faba* are extraordinarily permeable to urea and their osmotic pressure rises rapidly when they are placed in solutions of urea. HÖFLER and WEBER (1926) found that ether doubles the permeability of plant cells to urea.

Another case which has received extensive study is that of the translocation of **carbohydrates in plants**. The accumulation of sugar in the cotton plant by the transition cells of the phloem is against a concentration gradient (PHILLIS and MASON 1935, and MASON and MASKETT 1928). The dextrose gradient is always toward the stem. The sucrose concentration in the mesophyll is low, while the maximum is in the fine veins and interned in the stem phloem. Only the sucrose concentrations are changed by diurnal fluctuations and in "ringing" experiments, which indicates that sucrose may be the transported form. They calculated that the transport in the phloem was from 20,000 to 40,000 times greater than the rate of physical diffusion. They state that materials travel in the sieve tubes by some process involving independent movement of solutes; that accumulation may be polarized and linked with the nitrogen metabolism.

Although these conclusions seem plausible, it is not certain that the same result might not be otherwise attained, as for example, by mobile equilibrium of hexoses and sucrose with a "threshold" value for hexose such as is found in the kidney.

It has been suggested that the same kind of process may take place here as in the intestinal epithelium, i. e., the formation of hexose-monophosphates, or "phosphorylation" as an intermediate step in the accumulation of sugar. Phosphates promote the absorption of sugar. Extracts of intestinal mucosa can phosphorylate glucose and galactose but not the other sugars, maintaining a positive differential gradient towards the accumulation of glucose and galactose. (VERZAR 1935, VERZAR and LASZT 1935, LASZT 1935, MATTHIEU 1935).

Monoiodoacetic acid inhibits the absorption of glucose but does not affect that of Na_2SO_4 (VERZAR and LASZT 1935). The accumulation can also be inhibited by phloridzin (WILBRANDT and LASZT 1933, ABDERHALDEN and EFFKERMANN 1934, LUNDSGAARD 1933, DONHOFFER 1935). These results indicate that there

is an intimate relation between absorption and metabolism rather than a purely physical process depending solely on a concentration gradient. WESTENBRINK (1935) showed that the absorption of glucose is hastened by pre-feeding with glucose, fructose or galactose, while the absorption of fructose or galactose is hastened only by pre-feeding with fructose or galactose. The author assumes that there is an increased formation of enzymes in the intestinal wall necessary for the absorption of larger amounts rather than that the CORI ratio numbers for speed of absorption of hexoses and pentoses are valid. MATTHIEU (1935) showed that various hexose-mono- and hexose-diphosphates are absorbed in the rat intestine about as rapidly as fructose but considerably more slowly than glucose of the same concentration. The influence of blood sugar concentration on the rate of absorption of glucose or xylose from ligated small intestines of anesthetized rabbits was not reduced by continued intravenous injections of the above sugars. Glucose was absorbed twice as fast as xylose (McDOUGALL 1934). LASZT (1935) found that the absorption of glucose was increased at pH 7.0 while that of xylose is unaffected by the pH. See also GELLHORN and MOLDAVSKY (1934).

The absorption of dextrose by the small intestine (CORI 1925, CUTTING 1934); of fructose from the blood (WIERZUCHOWSKI and FISZEL 1935); of fructose by the liver, and other sugars such as glucose, maltose and galactose by the liver, intestinal tract, muscles and head (WIERZUCHOWSKI and FISZEL 1935b); the blood sugar regulation after intravenous administration of large doses of glucose (FENICIA 1935); the comparative rates of passage of hexoses from the blood to the tissues before and after blocking the reticulo-endothelial system (FIESSINGER and MARTINETTI 1935) and many studies on the concentration curve of glucose injected into, for example, the humoral artery and recovered from the vena mediana (DOGLIOTTI and TAGLIONI 1934) are too specialized to be more than mentioned in this monograph.

In the experiments of HÖBER and HÖBER (1937) the absorption of organic solutes by the small intestine of the rat was followed for various groups of reagents.

In the series of polyhydric alcohols, the absorption rate falls off with rising molecular volume. The absorption of aliphatic acid amides is analogous to that of the polyhydric alcohols except that the amides are absorbed more slowly than the alcohols of

corresponding molecular volume. Substances of distinct lipid solubility are absorbed at a higher rate than lipid-insoluble substances. Amino acids are absorbed at a higher rate than could be expected from their calculated molecular volume.

In offering an explanation of the behaviour of some sugars and amino acids in the intestine, the question has been raised whether an intermediary such as phosphorylation and dephosphorylation is concerned. HÖBER and HÖBER believe that this process cannot be responsible because iodoacetate works as an inhibitor of absorption. This conclusion of course depends upon the concept that the block produced by iodoacetate, in the oxidative chain, comes previous to the time when PO_4 enters into the picture.

The absorption of glucose and other sugars has been extensively studied in various connections with plants, animal tissues and red blood cells. See Reviews by JACOBS, HÖBER, for animal cells, etc., and ILJIN (1928) for plants.

The question of the distribution of sugar between plasma and corpuscles in animal and human blood has received considerable study. SVEDBERG (1933) has given a summary of this. In former determinations the interference of non-reducing sugars was always the stumbling block in blood sugar determinations. The problems of blood corpuscle permeability to glucose includes two questions: can blood corpuscles take up glucose? how does glucose penetrate into corpuscles? (See GRIJNS 1896; ANSELMINO and HOENIG 1930.) It is believed by some that the erythrocytes of animals except those of man, monkey and dog are impermeable to glucose. Chemically, human corpuscles were found to take up glucose (KOZAWA 1914, EGE 1920).

More recently OLMSTED (1935, 1936) has tried to solve this problem. He states that no glucose was found in certain species of erythrocytes when proper precautions in handling them were used. He finds that the addition of oxalates, the use of ether, insulin or adrenaline makes them permeable to glucose. This has been questioned by NEUWIRTH (1936), claiming to have used all of OLMSTED's precautions. It would be difficult to evaluate just how far such precautions were precisely followed. KLINGHOFFER (1935) using osmotic experiments concluded that red blood cells are permeable to glucose below a concentration of 2.3%.

The ratio at equilibrium of various non-electrolytes distributed between human red blood cells and oxalated plasma has been investigated by Wu (1922) and shows a lack of uniformity in the distribution coefficient of these substances. The results are illustrated in Table XVI.

Table XVI. The equilibrium relation between r. b. c. and plasma

	Concentration mg/cc		Ratio cells/plasma
	Cells	Plasma	
Urea	17.1	19.3	0.89
Glucose	99.9	103.2	0.97
Uric acid	1.93	3.92	0.49
Creatinin	2.48	1.24	2.0
NaCl	309.7	615.2	0.50
	(After Wu) (1922)		

SVEDBERG (l. c.) states that the existing data on blood corpuscle content of and permeability to glucose suggest the following theory: blood corpuscles in general absorb glucose. In most species there is no evidence of glucose permeability *in vitro*, but this fact does not exclude a slow penetration, balanced by oxidation *in vivo*. Man and possibly other species have blood corpuscles which are permeable to glucose.

The experiments of M. M. BROOKS (1934) on spectrophotometric analysis of rabbit's blood showed the rapid change from methemoglobin to oxyhemoglobin on intravenous injections of glucose. In this work the sudden change in color from the brown of methemoglobin to the red of oxyhemoglobin when glucose was injected can also be seen in the opposite ear vein and the simultaneous recovery of the animal noted. There is no question in this case that the erythrocytes are permeable to glucose, but since the cells are in an abnormal condition this result may be attributed to this state. However, since most of the studies made on the penetration of nonelectrolytes including glucose, were done *in vitro*, they therefore were done under abnormal conditions. These include such practices as the use of physiological saline solutions (which were formerly considered to be 0.85% and more recently 1.12% (See PONDER 1934) instead of serum;

or changing the pH inadvertently. SCRIBNER and RAPOPORT (1930) show that H_2CO_3 increases the permeability of the erythrocyte to glucose, thereby showing an H-ion effect. Changes in the H-ion concentration caused by handling or other methods, may be one of the factors responsible for the different results of various investigators. Just how far all these probable causes affect the results remains a question.

GELLHORN (1927) has colored eggs of *Holothuria tubulosa* and *Strongylocentrotus* with neutral red and noted that HCl, NaOH, glucose and several other sugars had no subsequent effect upon the color of the eggs, but when one of the non-electrolytes was combined with either NaOH or HCl the color changed to yellow. This change was more pronounced with NaOH than with HCl. The significance of this points to an increase in permeability due to change in osmotic pressure. This experiment is similar to that of SCARTH (1926).

SCARTH (1925, 1926) subjected *Spirogyra* to solutions of eosin and acid fuchsin in different concentrations of sucrose, dextrose and glycerine and by so varying the external osmotic pressure he was able to increase the speed of penetration of these dyes. He took the time required to produce a pink color in the nucleus or sap of 50% of the cells, and found that the time decreased when the osmotic pressure was increased or decreased from the normal to hypotonic or hypertonic. A strongly hypertonic sugar solution enables the acid dye, eosin, to penetrate into cells of *Spirogyra*. If the sap is acidified by acetic or citric acid, the cells acquire a strong affinity for acid dyes. He regards the cells as not injured under the more favorable conditions, when no mechanical disturbance of the cell takes place and believes that the increase in rate of penetration indicates an increase in permeability of the protoplasm. He regards this as disproof of the hypothesis that permeability is merely determined by partition coefficient between protoplasm and water. It is not because the solubility of dyes in protoplasm is low that they fail to enter, but he believes that there is an organized film on the surface of the cytoplasm which regulates permeability.

GELLHORN (1927) has investigated the effects of non-electrolytes on fertilization of sea urchin eggs in sea water. When HCl was added with glucose no fertilization took place. Nothing is said about whether the sea water was specially treated and

shaken free of the CO_2 which would be freed by the addition of HCl. CO_2 may increase the permeability or affect the rate of entrance of other substances. This effect may be an injury effect produced by the larger accumulation of CO_2 . It did not show up in ten minutes, but after 45 minutes there was a considerable decrease in number of eggs fertilized, thereby showing that the effect of the external medium had had time to act upon the eggs. When frog muscle is used the presence of cane sugar hastens the uptake of basic dyes (HIRUMA 1923).

The structure of gelatin gels from studies of diffusion by FRIEDMAN (1930) seems to throw some light on some possible causes for the action of sucrose and various non-electrolytes in causing an increase in permeability as found by SCARTH and various other investigators.

These authors found that glucose increased the rate of diffusion of urea into gelatine by 10% while sucrose increased the rate of diffusion over 32%. He found that those gels which had set at a lower temperature were more permeable to molecules than those set at a higher temperature, and interpreted this to indicate a more open structure. It has been further shown (FRIEDMAN 1930b) that the rate of diffusion in agar gels decreases linearly as the concentration of gel increases. These results are suggestive as plausible interpretations of the effects of non-electrolytes on the penetration of certain dyes.

The experiments of RANDALL and FAILEY (1927) on activity coefficients of non-electrolytes in aqueous solutions, the salting out of the ions, the relation between the activity coefficient and the dielectric constant (BRONSTED and WILLIAMS 1928) are all important leads in finding out reasons, for example, of the more rapid uptake of certain dyes by living cells in the presence of non-electrolytes.

The main experiments which have been done with animal cells are those with red blood cells, *Arbacia* eggs and cartilage cells. MOND (1930) has done semi-quantitative work on the rate of hemolysis of red blood cells of geese and humans. The pore diameter in geese is larger than in man, however, he believes that glucose penetrates human cells but not goose cells. Therefore he generally favors the lipid theory with the reservation that molecules with an MR_D less than 26 may penetrate according to the difference in size. MOND and HOFFMAN (1929) have used

cartilage cells of the frog in some semi-quantitative studies on penetration by means of the plasmolysis and recovery method. They found that in the spring the cells were more favorable to interpretation by the lipid theory whereas in the winter the pore theory was more important.

The direction of the passage of glucose through frog skin has been investigated by WERTHEIMER (1927) and is of interest in interpreting the factors responsible for penetration. Glucose will go inwards from isosmotic glucose to RINGER's solution but not outwards; it will go outwards from isosmotic glucose in distilled water but not inwards. When the H-ion concentration is increased and there is a lack of electrolytes, the membrane becomes permeable. It would appear that in this experiment the combination of non-electrolytes and electrolytes is not a conclusive proof of the conditions responsible for the penetration of non-electrolytes alone, as the charge on the membrane plays an important role.

A great deal of work has been done on the excretion and secretion of glucose and urea by the kidney. There is an apparent diffusion against the concentration gradient of these substances in the blood as compared with the kidney. HINSWORTH (1931) has shown that glycaemia occurs when the threshold for blood glucose is beyond 0.16%. The normal urine glucose is less than 0.1% (= 0.006 M) while the serum glucose is about 0.3%. The difference may be due to a possible back secretion by tubule epithelium (HERZFELD 1932). HÖBER (1934) showed that the concentration of urea in the urine is greater than that in the portal vein, and concludes that tubule epithelial cells accumulate and secrete urea.

RICHARDS (1929) has demonstrated in frogs the presence of glucose in the glomerular filtrate and its absence from the urine. A renal threshold is maintained in which the concentration of urinary sugar is independent of the diuresis, but proportional to the excess over the renal threshold of the blood-sugar concentration. The tubules appear to be the seat of the mechanism and the controlling factor is the sugar content of the blood in the peritubular capillaries.

HAMBURGER (1924) showed a differential permeability of the glomerular membrane to different sugars.

Insulin, according to BUFANO (1934), definitely increases the permeability of the kidneys during renal diabetes and slightly increases the permeability of normal kidneys.

MOORE¹⁾ found that the wall of the blastula of *Dendraster excentricus* acquires unusual properties of semi-permeability at about the time of the 10th segmentation. It remains freely permeable to water and to the salts of sea water, but is very slowly permeable, if at all, to sucrose, dextrose and other large molecules. A solution consisting of 2 to 9 parts of sea water and 1 part of M/1 sucrose causes immediate collapse of the walls of blastula and gastrula of *Dendraster*. Conversely, if the larvae are reared in such a sea water-sucrose solution, when they are returned to sea water after the 10th division the blastocoele immediately enlarges to twice its normal volume as the result of the osmotic tension developed by the included sugar molecules. The larva remains alive and continues its development.

BEEBLESTONE (1930) has studied the penetration of various carbohydrates including sugars into yeast, and found practically no difference in the osmotic reactions of these substances. Instead of using equal molality, he used equal percentages of substances. His Table II for example, shows that equal percentages of dextrans, di- and monosaccharides, and wort have identical osmotic effects, irrespective of the concentration of the carbohydrate and other molecules present. This is of course contrary to the original work of VAN'T HOFF, who first showed that isosmotic solutions are of substantially equal molality. No explanation is evident as to why these sugars all function osmotically alike. Further work is needed.

See also RESÜHR (1935) for penetration of certain non-electrolytes into unfertilized *Fucus* eggs.

Respiratory poisons which prevent oxidation of keto-acids derived by deamination of amino-acids by the kidney stop urea accumulation into the urine (HÖBER 1934). LAUG and HÖBER (1936) state that the perfused frog kidney concentrates the thiocyanate ion by secretory activity of the tubules, that it dilutes the bromide ion by resorptive activity of the tubules, but reacts indifferently toward the iodide ion.

From the foregoing experiments it might be concluded that in cases of simple, non-glandular cells, equilibrium is probably at equal activity. In glands there are other factors of metabolism and energy relations involved, associated with active

¹⁾ Personal communication.

cellular processes in addition to diffusion. Rates can be determined only on simple cells.

Rates

(The rate of penetration of non-electrolytes is more rapid than that of electrolytes. This was first established by JANSE (1887) for non-electrolytes other than water, which included KNO_3 , NaCl and sucrose in studies on plasmolysis and recovery. DE VRIES (1889) showed this for urea, and OVERTON (1899) for several hundred lipid soluble substances.

In the study of the rate of penetration of most non-electrolytes a graded series of homologs can be used with similar chemical properties but seriated as to polarity or to molecular size. A better correlation with the results is obtained when molecular refraction is used instead of molecular volume as a criterion. The molecular refraction (MR_D) is calculated either from the specific refraction of the substance or by summing up their atomic refractions at wave length D (NERNST 1926). The correlation between the molecular volume and the diffusion rates is not so satisfactory since the values for MV refer to pure substances without reference to aggregates or adherence of water molecules.

The criterion of polarity may be chemical in which the CH_2 chain has known substitutions, and the effects of these changes followed, or it may be based on its solubility in a non-aqueous apolar medium or its chemical reaction with the apolar medium. The several theories for the rate of penetration of non-electrolytes have as their basis of interpretation the correlation between the chemical and physical properties of non-electrolytes and the permeability constant. These are the pore or ultra-filter theory of RUHLAND (1912) in which the rate is correlated with molecular size of the penetrating substance; the lipid theory of OVERTON (1890); and the surface-film theory which postulates that there is a continuous oil layer over the whole surface of the cell, according to OSTERHOUT, DANIELLI and HARVEY (1934) and CHAMBERS (1935). Recent experiments of CHAMBERS and KOPAC (1937), indicate that the oil layer, if present, is not continuous.

The hemolysis method of studying the rate of penetration of substances into red blood cells is extremely simple and considerably accurate. JACOBS (1930) and JACOBS and STEWART

(1932) have described a method by which the cell was illuminated and the moment of hemolysis measured accurately even when the total duration of the experiment is only a few seconds. JACOBS (1931) concludes from the study of the penetration of a number of non-electrolytes into erythrocytes that two paths are available depending either upon the relative size of the molecules or their lipid solubility. If the molecules are sufficiently small they will enter the cell regardless of their lipid solubility; if they are sufficiently lipid soluble they will enter the cell regardless of their size. These theories depend upon the observations made with artificial membranes in which the pore size can be altered and upon the results obtained with the relative lipid solubility of substances. When the series is homologous, molecular volume is a determining factor (HÖBER and ORSKOV 1933). When the series is not homologous, the chemical nature of the solute or the plasma membrane or both appears to be the dominant factor (ULRICH 1934, HÖBER and ULRICH 1934). The quantitative experiments of PARPART and SHULL (1935) on the penetration of glycerol into ox erythrocytes compares with the results obtained by JACOBS (1931).

In the earlier experiments, not sufficient data were given to assign absolute values in terms of the permeability constant, P . The general form of the equation used in determining this constant is that of FICK's diffusion law, by which it is necessary to know the quantity of material which passes in unit time across a known area and thickness of membrane and which is proportional to the concentration difference across the membrane. A table has been devised equating to common terms in gm. mol/cm⁻²/sec (GM/l) the various values for P from the data of JACOBS, NORTROP, HÖFLER, FUJITA and MICHAELIS, COLLANDER and WILBRANDT for comparative purposes. These data are summarized in Table XVII. In the later experiments, such data as the area of the cross section through which the substance passes and the thickness of the membrane have been included where these values are given.

Artificial membranes

Artificial membranes afford an excellent means for the study of the pore theory. In these membranes, the pore diameter is determined by POISEUILLE's Law, and shows a correlation between

Table XVII. Permeability Constants for Certain Non-Electrolytes

Organism	Tissue or Cells	External Solution	t° C	P	Observer
Arbacia	Unfertilized Eggs	Ethylene Glycol 0.5 M	22	$M/cm^2/sec/M/l \times 10^{-7}$	STEWART and JACOBS (1936)
	Fertilized "	" "	"	0.0734	
	Unfertilized "	Diethylene "	"	0.1468	
	Fertilized "	" "	"	0.0433	
	Unfertilized "	Propylene "	"	0.0866	
	Fertilized "	" "	"	0.1280	
Rheo discolor .				0.2560	BÄRLUND (1929)
	Epidermis	Urea	20	$M/sec \times 10^{-7}$	
	"	Lactamide	"	141.	
	"	Thiourea	"	297.	
	"	Methyl urea	"	355.	
	"	Glycerol	"	494.	
Gentiana sturmi- ana	Red epidermal cells	Urea	10.5	1100.	HÖFLER (1930)
				$M/sec \times 10^{-7}$	
Chara cerato- phylla				$M/sec \times 10^{-7}$	COLLANDER and BÄRLUND (1933) ¹⁾
	Whole cell	Butyramide	—	3,300—16,500	
	" "	Propionamide	—	cm/sec $\times 10^{-7}$	
				470.	
				360.	

	"	Acetamide	—	36.	HÖFLER (1930)
	"	Ethylene glycol	—	120.	
	"	Glycerol	—	21.	
	"				
Majanthemum . .	Epidermal cells	Urea	—	$M/sec \times 10^{-7}$	
		Thiourea	—	390—555.	
		Methyl urea	—	1500.	
		Lactamide	—	1250.	
		Glycerol	—	470.	
		Malonamide	—	164.	
		Various sugars	—	91.6 5.5—14	
Dried collodion	—	Urea	18	$M/cm^2/sec/M/l$ $\times 10^{-7}$	NORTHROP (1929)
	—	Sucrose		0.00055	
	—	Glycerol			
Gelatin	—	Sucrose	5	$M/cm^2/sec/M/l$ $\times 10^{-7}$	FRIEDMAN (1930)
	—	Glucose	5	0.152	
				0.304	

¹⁾ Recalculated from Table 15, p. 106.

the approximate size of the pore and the molecular size of the penetrating substance. If the pore is less than 7×10^{-1} cm., POISEUILLE's Law cannot be applied because the substance is considered to be equivalent to a solid.

In experiments with wet and dry collodion, the difference in the size of the pores of the two kinds of membranes formed, can be utilized for comparative results. In dry collodion the diameter of the pores is smaller than 7×10^{-7} cm. while in wet condition it is larger than this. When freshly made collodion membranes are immersed in water before the complete removal of the organic solvents, the membranes are permeable for all substances of small molecular weight. If the membrane is allowed to dry, either before or after placing in water, the membrane exhibits in general the same type of permeability as living cells (NORTROP 1929). The mechanism of the permeability of the "wet" membranes was found by DUCLAUX and ERRERA (1925) who showed that the relative rates of flow of various liquids through these membranes was in proportion to the viscosity of the liquids. There seems to be no doubt that these membranes consist of a network of capillaries through which the solutions pass. As the percentage of water held in the membrane decreases, the size of the pores decreases, until with membranes containing 5 per cent or less of water, the rate of flow of water is too small to be measured and the pore size cannot be determined. In some experiments on the penetration of substances through dried collodion, NORTHROP (1927) showed that the rates of penetration of sucrose, urea and glycerine were all essentially alike, namely, less than 2×10^{-7} GM cm⁻² hr⁻¹ (GM l⁻¹)⁻¹ even though the molecular weight of the substances used differed. In these cases the hypothesis would indicate that the pore diameter is greater than that of the diameter of the molecule of the substances used.

COLLANDER (1926) used three membranes of different pore size and showed a correlation between the relative permeability of the membranes and the molecular refraction of the penetrating substance. He assigned an arbitrary value of 100 for the rate of penetration of ammonia across the membrane having the largest pore size, and compared the rates of other substances with this. There was good agreement between the molecular volume and the rate of penetration, and the pore size and the rate of penetration. FUJITA and MICHAELIS (1926) used both wet and dry membranes,

the former having wide pores and the latter having pores the size of a molecular sieve. When organic non-electrolytes are used, there are discrepancies in the relative permeability of the membranes both from the point of view of M. W. and that of MR_D , but in general the rate of penetration follows the usual order, i. e., it decreases inversely as the molecular size. This is the usual order in the case of free diffusion, and would indicate that in those cases where there is agreement, the membrane acts by virtue of this property. When the pores are large, there is little difference in the permeability rates, but when the pores are molecular, they are impervious to substances having a higher MR_D value than around 40. It is obvious therefore that the size of the pores in these experiments with collodion is the important factor. There is one essential difference, however, between collodion membranes and living cells, namely, the thickness of the membrane. This is indicated by the extreme slowness in which substances pass across the membrane, as compared with the speed of reactions in the living plasma membrane.

If gelatine or agar is mixed with formalin according to FRIEDMAN (1930) and FRIEDMAN and KRAEMER (1930), the pore diameter becomes about 7×10^{-7} cm. With a thickness of 0.15 cm. the value for P in the case of the penetration of sucrose is 550×10^{-7} and that for glucose, 100×10^{-7} $\text{GM} \cdot \text{cm}^{-2} \text{hr}^{-1}$ ($\text{GM} \cdot \text{l}^{-1}$)⁻¹. In other words, in this case, there is a difference in the rate which varies inversely with the molecular weight of the substance penetrating. According to ULRICH (1934) the permeability of the erythrocytes of different animal species shows certain fundamental differences which contradict the lipid ultrafilter theory. He studied the penetration of lipid-insoluble non-electrolytes, such as pentoses, hexoses, hexitols, disaccharides and amino acids of relatively large molecular volume into mammalian erythrocytes. The results indicate the existence of specific affinities of the cell membrane for the penetrating molecule. MOND (1930) on the other hand attributes the relative permeability to levulose of avian and human erythrocytes to lipid solubility rather than to pore diameter.

The concept that there is a continuous layer of an oil over the surface of a cell has been given by DANIELLI and HARVEY (1935) as the explanation of their experiments on surface tension measurements with eggs and artificial systems. They concluded

that the surface of the cell is built up of a layer of an oil which by virtue of its relatively low surface tension gives fairly coherent films. It might be mentioned, however, that this concept does not receive support from the experiments of CHAMBERS and KOPAC (1937) who addressed a minute oil droplet onto the surface of a sea urchin egg and noted that it did not fuse with the surface but slipped intact into the interior of the egg. Had there been a continuous lipid layer on the surface the drop would have spread out in the lipid,

Plants have been used to study the penetration of non-electrolytes by the plasmolytic method and subsequent recovery. HÖFLER (1930) used the red-violet epidermal cells of *Gentiana* which show a high permeability for urea. By measuring the area and volume of the protoplasm inside, the critical plasmolysis and the concentration of the external solution at intervals, he found that the cell took up from a concentration of 1 M urea .02 to 0.1 grams per minute. The other parts of the stem were less permeable.

BÄRLUND (1929) using the same method of plasmolysis calculates the rate of penetration of several non-electrolytes into cells of *Rhoeo* by FICK's Law, as follows:

$$P = \frac{1}{t} \ln \frac{C}{C-x}$$

in which C is the concentration of the external solution and x, the concentration of the internal solution at time, t. Since no data for area and volume are given, absolute units cannot be calculated, but the relative values for permeability according to the above formula are given in Table XVIII. When values for MR_D are included, the agreement is not very general.

Table XVIII

Substance	MR_D	P
Urea	13.67	.051
Lactamid	21.01	.107
Thiourea	19.59	.128
Methyl urea . . .	18.51	.178
Glycerine	20.63	.400

After BÄRLUND (1929).

WILBRANDT (1931) used the same method and plant as BÄRLUND and obtained a fair agreement with his results. He gave the area and volume on *Rhoeo*. The minimum rate of penetration was that for sucrose in which $P = 0.035 \times 10^{-7}$ and the maximum EtOH and MeOH in which $P = 70.6 \times 10^{-7}$. He shows that the P constant increases as the MR_D decreases, but the deviations from the general results cannot be explained by any one theory.

The chemical relations of the penetrating non-electrolytes having NH_2 or OH groups have been emphasized by WILBRANDT (1931) using the plasmolytic method in three plants, *Rhoeo*, *Begonia* and *Basella*. The substances were chemically but not physically related. The permeability ratio between *Rhoeo* and *Basella* for the rate of penetration of glycerine, for example, was 1/1, while that between *Rhoeo* and *Begonia* was 50/1. When urea was used, the ratio for the former two plants was 1/80 and for the latter 1/1. There may be present in this case a possible correlation with acid or basic dissociation of the non-electrolyte. These differences cannot be explained by either the pore theory or the lipid theory as it stands, without a further knowledge of the essential differences in the protoplasts of the plants themselves. See HÖBER (1936) for further details on theories of penetration.

SCHÖNFELDER (1930) used the cells of *Beggiotoa mirabilis* which is noted for its large pores. There was a parallelism between the rate of penetration of the non-electrolytes and their molecular volume in general, but those which are lipid-soluble have an additional advantage in penetrating. In the experiments of COLLANDER and BÄRLUND (1933) the analysis of the sap of *Chara ceratophylla* into which non-electrolytes were allowed to penetrate showed a ratio of substance inside to that outside of 90 to 100%, and in some cases, to 70%. The data are calculated on the basis of FICK's Law in which volume is given and the area of the cell. He concludes that the plasma layer is of the magnitude of $.3 \mu$; that there is a general correlation between lipid-soluble substances and their speed of penetration. The exceptions may be attributed to the size of the pores or to the molecular volume of the diffusing substances.

The permeability of *Spirogyra* and *Elodea* to urea were investigated by WEBER (1931, 1932). The absorption of non-electrolytes by the cells of *Hookeria lucens*, *Hydrodictyon utriculatum*,

and *Basidiobolus renarum* was investigated by BONTE (1935) who concluded that lipid solubility was a factor in penetration more so than that of molecular volume.

In the case of phanerogamic plants, plasmolysis and recovery, as initiated first by KLEBS (1887, 1888) and DE VRIES (1888) have been used by WIELER (1887), ILJIN (1928), and more extensively by HÖFLER (1926). ILJIN observed recovery from plasmolysis in sugar solutions and that the presence of salts in the external solution hindered the exosmosis of mono- and disaccharides in *Allium cepa*. A summary of the older literature is given. HÖFLER showed that the uptake in gm. mols/liter/hour was greatest in the case of maltose for both *Gentiana sturmiana* and *Maianthemum bifolium* according to Table XIX.

Table XIX

Substance	Uptake in gm. mol/liter/hour	
	Gentiana $\times 10^{-5}$	Maianthemum $\times 10^{-5}$
Sucrose 0.6 M .	60—90	100—160
Glucose . . .	60—100	160—413
Fructose . . .	110—200	200—570
Maltose	190—307	240—550

After HÖFLER¹⁾ (1926).

Arbacia eggs have been considered more initially normal than red blood cells because of their normal environment during experimentation. The permeability constant for such non-electrolytes as ethylene glycol, diethylene glycol and propylene glycol for *Arbacia* eggs has been found by JACOBS and STEWART (1932) to be of the same order of magnitude as that for ox erythrocytes. (See Tables, STEWART and JACOBS 1932, 1936).

The permeability constant for the penetration of certain non-electrolytes into unfertilized eggs of *Arbacia punctulata* was obtained by McCUTCHEON and LUCKÉ (1928) who showed that permeability was greater in hypotonic solution of dextrose, saccharose and glycocoll than in sea water of the same osmotic pressure.

¹⁾ The area of the cell surface is not given.

Another as yet unexplained problem is that arising from the study of species differences of red blood cells in relation to the penetration of various non-electrolytes. Why for example, does the blood of the higher apes and man have a different permeability constant to glucose than that of the lower laboratory animals? Why is hemolysis to ethylene glycol, for example, very rapid in one group of mammalian erythrocytes and very slow in another? Why does the erythrocyte of the rat resemble, for example, that of the mouse in its permeability to one substance and is quite different from it in its permeability to another substance? Such are examples of unexplained facts which offer a fascinating study and seem to defy as yet all theories. See JACOBS (1931), JACOBS, GLASSMANN and PARPART (1938), and OLMSTED (1936) for further discussion on species differences.

In concluding this chapter on the penetration of non-electrolytes, there are several generalities which seem to apply in those cases where the facts are known. The permeability for small molecules seems to be controlled, first, by either the molecular volume of the penetrating substance which is also affected by the differentiation in size of the pores in the membrane, or second, by the lipoid solubility of the substance penetrating. The latter hypothesis is based upon the assumption that there is a layer of lipoid either continuous or discontinuous in the plasma membrane. When larger molecules penetrate their lipoid solubility is invoked. Other hypotheses such as a mosaic of lipoid and protein in the plasma membrane may account for hitherto unexplained results, such as those obtained by chemical rather than physical factors of the penetrating substances.

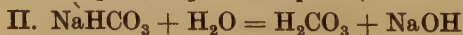
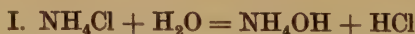
CHAPTER X

PERMEABILITY TO WEAK ELECTROLYTES AND GASES

Weak electrolytes

The study of the penetration of weak electrolytes has been largely confined to a few weak acids and bases including fatty acids, amino acids and alkaloids. Such organic acids as malic, succinic, tartaric, lactic, pyruvic, citric, oxalic and carbonic have been produced by metabolism and are found in various cells. The bases produced by the breakdown of amino acids or proteins, such as asparagine, glutamine, purine and so on are converted into Me_3NHOH and finally into NH_3 . They also diffuse through the cell. A proper balance between these various factors is necessary for the maintenance of life. These substances are usually included under the heading of weak electrolytes, having a pK around 2.0. It has been demonstrated by direct measurements that such weakly dissociated acids and bases as CO_2 and NH_3 enter the cell readily. Table XX gives dissociation constants for certain ones.

A consideration of the mechanism of penetration of weak electrolytes raises the question of the penetration of ions versus molecules. (Molecules penetrate relatively fast, whereas ions exchange for other ions, and have heretofore been considered to penetrate relatively slowly.) We may consider Phase III as discussed in the chapter on Strong Electrolytes as the period of accumulation comparable with that of accumulation of the ions of weak electrolytes. Weak electrolytes, because of their high concentrations of undissociated molecules, have the property of accumulating in living cells. The explanation of the mechanism is as follows: hydrolysis of the substance takes place so that, for example,



and NH_4OH or H_2CO_3 will penetrate as molecules. The concentration of molecules present will depend upon the H-ion concentration of the solution reacting. If the pH of the cell interior is different from that in the external solution, the molecules, on entering, will dissociate either more or less according to the pH of the interior. If further dissociation takes place inside, then as the ions are removed, more molecules will come in until an equi-

Table XX. Dissociation constants of some weak bases and acids

Substance	K_b
Bases	
Caffeine	4.1×10^{-14}
Cocaine	4.0×10^{-7}
Quinine	2.2×10^{-7}
Strychnine	1.0×10^{-7}
Ammonia	3.6×10^{-5}
Acids	
Arsenious	6.0×10^{-10}
Benzoic	6.6×10^{-5}
Boric	6.4×10^{-10}
Butyric	1.48×10^{-5}
Carbonic	3.0×10^{-7}
Uric	1.5×10^{-6}
Hydrocyanic	7.2×10^{-10}
Alanine	9.0×10^{-10}

brium has been reached with the outside. (The rate of penetration and the final equilibrium attained are a function of the dissociation constant.) There is therefore inside the cell, in addition to the molecules in equilibrium with those outside, also the ions which have been produced by dissociation, and are trapped inside, so that the total concentration inside is greater than that outside. This is the mechanism of accumulation, or "trap" mechanism of the penetration of weak electrolytes. (See also McCutcheon and Lucké 1924.)

The protoplasm of the cell is probably buffered around pH 6.5 to 7.0 according to the studies of various investigators. Since acid solutions have a lower pH than this, weak acids on entering

would become more dissociated, giving rise to H ions.. CHAMBERS (1928, 1929) injected acids and found that cells would not tolerate more than a certain change in pH, and any material change in pH resulted in injury. Since molecules penetrate fast, equilibrium is considered to be attained when the molecular concentration inside and outside are equal.

The importance of the molecule was first recognized by KLOCMAN (1911) who compared the disinfecting action of acetic acid alone, and in combination with small amounts of HCl to depress its dissociation, concluding therefrom that the molecule is effective as well as the H-ion. HARVEY (1911) showed that weak acids and bases enter sea urchin eggs readily; VAN DAM (1918) in a study of the souring of whey, found that the organisms were fairly resistant to the H-ion, and to Na-lactate, but are very sensitive to the undissociated lactic acid molecule; LOEB (1913) states that the power of acids to initiate development in certain unfertilized marine eggs cannot be due to the H-ion, for tartaric, citric and the mineral acids are relatively ineffective as compared with the fatty acids, and concludes that the action is molecular rather than ionic. When salts of the fatty acids are added to the external solution, the resulting mixture is more toxic to infusoria than the solution without the salts. Since the addition of salts depresses the dissociation of these acids, thereby increasing the concentration of molecules in the outside solution, the greater toxicity may be explained on this basis (COLLETT 1921, LOEB 1913, LILLIE 1926, 1927). COLLETT (1921) made a study of the toxicity to *Paramoecium* and *Euplotes* of several organic acids, using both alone and in conjunction either with their sodium salts or with HCl. She identified the factor responsible for toxicity as the undissociated molecule which penetrates and then dissociates inside releasing the H-ions. SMITH (1924) showed the influence of CO_2 on the velocity of division of marine eggs. In WERTHEIMER's experiments (1924) on the penetration of weak acids into frog skin he states that these acids do not follow the degree of dissociation nor the lipid solubility of the acid penetrating.

Weak acids. Experiments illustrating the effects of the hydrolytic dissociation of NaHCO_3 have been done by JACOBS (1920) with *Symphytum peregrinum* petals. He concluded that the rapid penetration of relatively large amounts of undissociat-

CO₂ is responsible for the rapid change in the color of the petals. POIJÄRVI (1928) used *Rhoeo*, *Tradescantia* and *Lemna* root hairs, and McCUTCHEON and LUCKÉ (1924) *Nitella* and sea urchin eggs; M. M. BROOKS (1923) showed that CO₂ and NH₃ enter *Valonia* readily; OSTERHOUT and DORCAS (1925) and JACQUES (1935, 1936) made a more quantitative study of the penetration of CO₂ and H₂S (Table XXI).

Table XXI. Permeability Constants
for Certain Weak Electrolytes

Organism	Tissue or Cells	Sub- stance	t °C	M/cm ² /sec/M/l × 10 ⁻⁷	Observer
Rana . .	Skin	CO ₂	22	0.0023	WRIGHT (1934)
" . .	Muscle	"	"	0.0040	
Cat . . .	Smooth Muscle	"	"	0.0037	
Dog . . .	Connective Tissue	"	"	0.0019	
" . . .	Striated Muscle	"	"	0.0035	
Sheep . .	Red Blood Cell	CO ₂	—	170	DIRKEN and MOOK (1931)
Valonia macro- physa .	Whole Cell	CO ₂ ¹⁾	—	2.5	JACQUES (1935—36)
KÜTZ .	" "	H ₂ S ¹⁾	—	2.2	

The early development of normally fertilized eggs of *Asterias forbesii* and *Arbacia punctulata* have been followed by quantitative methods under conditions of varying CO₂ tension, H- and HCO₃-ion concentration in sea water by SMITH and CLOWES (1924). They concluded that CO₂ is able to penetrate through channels which do not involve ionic equilibrium.

Carbonic acid is the most effective of any weak acid studied when pure solutions of equal pH are compared. Since the dissociation constant of this acid is lower than that of other acids, there are more undissociated molecules present. This undoubtedly accounts for the rapid changes in internal pH of the cell.

¹⁾ The observer assumes that these substances penetrate in the molecular form and hence they are listed as gases.

Former views concerning the specific effect of carbonic acid as not due to H ions can now be dismissed. The experiments of JACOBS (1920), who found that a saturated solution of CO_2 was more toxic to tadpoles than are solutions of oxalic, formic, butyric or acetic acids of the same H-ion concentration, can be so explained.

In experiments with strong acids, the presence of bicarbonates either in the external solution or in the cell wall tend to confuse the picture. As the bicarbonates break down under the action of the acid, CO_2 is liberated. This diffuses very rapidly into the cell, and unless eliminated in the final result as was done by M. M. BROOKS in the case of *Valonia* sap, the change in pH of experiments utilizing either the natural indicator or the introduced indicator might be attributed to actual penetration of the acid being studied, rather than to the secondary effects of the CO_2 .

M. M. BROOKS (1923) using *Valonia ventricosa*, studied by direct determinations of the pH of the sap, the rate of penetration of various acids through protoplasm, taking this factor into account. It was found that strong acids decompose the bicarbonates of the cells. The CO_2 diffuses into the sap and can be removed by suction so that changes in pH not due to the CO_2 can be observed. It was found in this work, as also in that of the previous investigators, that weak acids penetrate rapidly, and in general the rate follows directly the concentration of undissociated molecules present in the external solution; i. e. (the weaker the acid, the more undissociated molecules present, and the more rapid the penetration.) Injury also occurs more rapidly in the case of the weak acids than with the strong acids, which is consistent with the relative rate of penetration of the two species of acids.

Most of the earlier workers on the penetration of weak electrolytes tried to find a correlation between the rate of penetration and the dissociation constant of the substance penetrating.

HARVEY (1915) used the pigmented gonidial filaments of a holothurian *Stichopus ananas* and noted the rate of change of color produced by a large number of acids including both strong and weak electrolytes. He concluded that there was no relation between the degree of dissociation of an acid and its toxicity, but that there is a general relation between penetrating power on the one

hand and lipoid solubility on the other. CROZIER (1916) made observations on a number of concentrations in a series of acids similar to those used by HARVEY. Although using an unrelated animal, *Chromodoris zebra*, his results in general paralleled those of HARVEY, and also show that speed of penetration of the acid increases with increasing concentration of acid.

HIND (1916) has used potato tubers and roots of *Vicia faba* seedlings. The external solution contained either weak acid or one of several strong ones. The absorption of the acid was followed by measuring the conductivity of the external solution. The conclusion is that exosmosis of electrolytes produced in the case of a mineral acid is considerably less than in the case of formic or acetic acids.

The activation of starfish eggs by **other weak acids** has been studied by R. S. LILLIE (1926, 1927). The readiness with which the acid penetrates the egg protoplasm is a chief factor in its physiological action. Activation is attributed to the penetration of undissociated molecules, and the dissociation of the molecules inside the egg, furnishing the H-ions which effect activation. LILLIE used organic acids widely different in molecular concentration but closely similar in C_H . In the case of each of the fourteen weak acids showing parthenogenetic action, the rate of activation proved nearly proportional to the concentration of acid. Carbonic acid is physiologically identical with the action of fatty acids, and the same proportionality between its concentration and rate of action is found as with the other weak acids.

The sense of taste has been used for determining the rate of penetration of various acids. A summary of the earlier work is given by TAYLOR (1928). RICHARDS (1898) and KAHLENBERG (1898) state that taste is due to the presence of H-ions. Acetic acid, which cannot be tasted at dilutions below N/200, is only 6 per cent dissociated at that concentration, whereas HCl, which is completely dissociated, is tasted down to N/900. From this observation it would seem that the H-ions were responsible for the taste. However, TAYLOR (1928) found that two acids with the same dissociation constant, namely, tartaric and acetyl lactic, have different degrees of sourness at equal concentrations. It would appear therefore, that the problem concerning the sense of taste includes also the study of the penetration of anions through the plasma membrane.

The effects of auxins or growth-promoting substances may be due to their ready penetration as a function of their property as weak electrolytes in addition to their probable specificity. See BOYSEN-JENSEN (1936). The substance isolated, β -indole acetic acid, is a weak electrolyte, and it has been shown by ALBAUM and others (1937) that its efficiency in penetrating cells of *Nitella* depends upon the concentration of undissociated molecules present.

A large number of dyes are weak electrolytes, but these are treated in a separate chapter, and will not be further referred to except in the case of a few quantitative studies. Using the dye 2,6-dibromo phenol indophenol, one of CLARK's oxidation-reduction indicators, M. M. BROOKS (1926) showed that this dye also followed the "trap mechanism" for weak electrolytes, and that there was a constant ratio between the undissociated molecules in the external solution and the dye in the sap. IRWIN (1927) using the dye trimethylthionine obtained similar results.

Amino acids do not enter tissues with great rapidity as shown by the earlier experiments of OVERTON (1902) with frog muscle. From the experiments of RUHLAND and HOFFMANN (1925) and of SCHÖNFELDER (1930) on *Beggiatoa*, which allows non-electrolytes to penetrate readily, it was also found that such amino acids as glycine, alanine, asparagine, and leucine penetrate very slowly. Since these substances are ampholytes one would expect them to penetrate more in accordance with the slow rates found for electrolytes. HÖBER and HÖBER (1937) have found that when interstitial loops were used to test the rates of absorption of such amino acids as glycine, asparagine and alanine, the rates were faster than those found with other organisms.

The permeability of tissues to proteins has been extensively studied, especially in relation to capillaries and several anatomical portions of the kidney in numerous publications by RICHARDS, A. N. MARSHALL, WHITE, which can be consulted in reviews such as that of MARSHALL (1934). See also GRÜNWALL (1935), CONKLIN (1935), BRULL (1934), MOSONYI and VOITH (1935). In this connection it is interesting to note the effect of pH upon the elimination of dissolved hemoglobin by the perfused frog kidney as determined by WEBSTER, ENGEL, LAUG and AMBERSON (1934) who found that the elimination was large at pH 5.5 and small at pH 7.8.

Weak bases

Since the rate of penetration of molecules is relatively fast as compared to that of ions, the rate of penetration of weak bases and their concentration in the cell at equilibrium will also depend upon the concentrations of molecules and ions in the solution. Therefore the dissociation constants of the substance used, and the pH values inside and outside the cell will determine the concentration in the cell at equilibrium. Ammonium has been used by a number of investigators to change the internal pH of the cell. The rapid diffusion of NH_4 and the consequent change in pH is a convenient method as well as relatively without injurious effects. (HARVEY (1911, 1914) mentioned the importance of dissociation constants as a factor showing that weak bases penetrated rapidly as compared with strong, and called attention to the relation between the rate of penetration and the degree of dissociation of the substances used.)

The favorite base which has been used, aside from the alkaloids and amines in experiments of this nature has been ammonia and its salts. WARBURG (1910) first showed that sea-urchin eggs stained with neutral red were entered readily by NH_4OH . It must be noted that neutral red does not stain protoplasm but only the cellular inclusions, which changed color. Since the NH_4OH had to pass through the protoplasm to reach the cellular inclusions, however, this is evidence that NH_4OH penetrates the protoplasm readily. HARVEY (1911) obtained similar results with living plant cells of *Spirogyra* and *Elodea*, and with *Paramoecium* and sea-urchin eggs. HOAGLAND and DAVIS (1923) showed that ammonium salts in .005 M concentration changes the cell-sap of *Nitella* from pH 5.2, which is normal for the western species, to 6.2 in the course of 24 hours or less. After this time the cells became injured and exosmosis of the chlorides occurred. Chlorides come out before sulphates or phosphates.

JACOBS (1922) showed by means of the cells of flowers of a *Rhododendron*, which contains a natural indicator, with starfish eggs stained with neutral red, and with frog skin used as an artificial membrane, that increased intracellular alkalinity may be brought about by solutions of ammonia salts. He noted that the pH attained for the inside of the cell depends not upon the pH of the external solution but on the absolute concentration of the free base in the external solution. He studied the penetration

of NH_4OH into various marine tissues and several fresh water organism and found that weakly dissociated alkali salts penetrate marine tissues almost instantly, while the strongly dissociated only after a comparatively long time.

M. M. BROOKS (1923) studied the penetration of ammonium into *Valonia ventricosa* and obtained quantitative measurements on the penetration of NH_3 by following the pH of the sap. This is very favorable material for this work since the central vacuole contains the cell sap which can be expressed and measured directly. Everything must go through the protoplasm in order to reach the sap, so that the protoplasm is permeable to all substances which are found in the sap.

In these experiments the pH value of the expressed sap was determined at various concentrations of NH_4OH in the external solution. Concentrations of NH_4OH from .0015 M to .03 M produced different pH values in the sap, very quickly attaining an equilibrium value from pH 8.8 to 11.5 and increasing in pH as the external concentration increased. In all these experiments the pH of the sap never exceeded that of the external solution except when the external pH remained at 10 owing to buffer action of sea water.

In explaining the penetration of ammonium, the possibility of considering penetration as due to dissolved gas molecules in the solution and the passage of electrically neutral particles is offered. This idea had been current in the literature as implied in the work of HARVEY and others cited in this chapter. OSTERHOUT (1925) offered this explanation in connection with the penetration of CO_2 or H_2S to account for the penetration of all substances into living protoplasm. The presence of ammonium in the sap was demonstrated by a heavy precipitate with NESSLER's reagent. The effects of NH_4Cl upon the penetration of arsenic into *Valonia* sap showed (M. M. BROOKS 1925) that the pH of the interior of the cell had a marked effect upon the amount of arsenic entering.

PORT (1926) found similar differences in effects of NH_4OH and alkali salts of the strong electrolytes in noting changes in color of the corolla of *Viola tricolor*.

MCCUTCHEON and LUCKÉ (1924) changed the pH of the sap of *Nitella*, starfish eggs stained with neutral red and *Gonionemus* larvae to a more alkaline reaction with ammonium salts in order

to show the effects of a change in the internal pH on the penetration of dyes. Their results showed that a more alkaline internal pH retarded the penetration of the basic dyes, brilliant cresyl blue and neutral red.

The same results were found by IRWIN (1925) with the sap of *Nitella*. Her explanation of the results assumed that the pH of the sap was independent of its NH_3 concentration. However, since this is contrary to experiments and to the Mass Law these results can be explained in the same way as by the "trap mechanism" of weak electrolytes.

STEWART (1931) showed the rapid rate of penetration of ammonium salts into *Arbacia* eggs.

SCHJØDT (1933) investigated the swelling of erythrocytes in solutions of ammonium salts. Time/volume curves produced by equations were developed and the author claims that the cells obey the BOYLE-MARIOTTE Law, but see PONDER (1934).

JACOBS and STEWART (1936) have used the criterion of swelling in their interpretation as to whether or not ammonium salts penetrate *Arbacia* eggs. They conclude that since the eggs do not swell in the presence of the salts of the strong acids, but do in the presence of the salts of weak acids, therefore, in the former case, penetration takes place very slowly, whereas in the latter case, penetration takes place readily. If swelling of the eggs is due to intake of water, then we might conclude that the rate of intake of water is hindered by the ammonium salts of the strong acids, but not by those of the weak acids. This effect on the penetration of water should not be confused with the penetration of the NH_3 itself. It has been shown in the first part of this chapter that this molecule penetrates a great variety of living cells, even when present as the chloride. In conformity with previous experiments there must also have been a change in the pH of the cell in the presence of any of the ammonium salts used. It is interesting to note that hydrolysis of the salts used should produce swelling in one case and not in another, i. e., that hydrolysis, in the case of the salts of the strong electrolytes should effect the intake of water adversely, but in the case of the salts of the weak electrolytes there is no effect. The effect of H-ion concentration on the toxicity of amines has been shown, for example, in such studies as those of HOWLAND and BERNSTEIN (1934) for *Amoeba proteus*.

While JACOBS (1931) believes that cells in general are not freely permeable to NH_4 ions, and that the erythrocyte is an exception, nevertheless it appears that the penetration of this substance as NH_3 or NH_4 or NH_4OH must follow the same laws as operate in the case of non-electrolytes. The salts of ammonium hydrolyze to form NH_4OH . The concentration of such molecules increases as the pH is increased, so that at higher pH values, more molecules should be found in the cell. According to FICK's Diffusion Law the concentration in the cell should increase until it equals that outside the cell, so that at equilibrium the concentration inside and outside should be equal unless there are other factors at present unknown to prevent this.

The only direct quantitative experiments known to the writers are those of M. M. BROOKS (1923) who showed that in the case of *Valonia* there is a definite relation between the concentration of NH_4OH in the external solution as compared with that in the sap at various pH values (concentrations) so that as the external concentration of NH_4OH is increased, the pH of the sap becomes more alkaline. That this alkalinity is due to the presence of NH_4OH was shown by heavy precipitates with NESSLER's reagent and removal with compressed air. The concentration of ammonium in the sap at various times and at equilibrium depended upon the concentration in the external solution.

An explanation of the ready penetration of both CO_2 and NH_4 or NH_3 may be found in the lipid-soluble areas of the cell.

The pharmacological effects of alkaloids as a function of pH. The experiments dealing with alkaloids can be satisfactorily explained by experiments showing that the toxicity of the substance depends upon the concentration of undissociated molecules present in the external solution. Since alkaloids come under the heading of weak electrolytes, any change in pH value would constitute a change in the concentration of the undissociated molecules present. This effect has been observed by various investigators.

OVERTON (1896, 1897) found that the salts of the alkaloids are less poisonous than the free base and that the free bases enter more quickly than their salts. See also CRANE (1921). LOEB (1898) found atropine to be less toxic for *Paramoecium* if in alkaline solution. Strychnine and veratrine were not affected by a change in reaction. PROWAZEK (1910), however, found alkaline solutions

of strychnine, atropine and quinine to be more toxic for *Colpidia* than acid solutions. Similar results were obtained by TRAUBE (1912) in determining the toxicity of these alkaloids for tadpoles. PŘIBRAM (1911) found that the hemolytic action of cocaine on red blood cells is greater in alkaline solution. In the case of plants, TRÖNDLE (1920) observed that quinine penetrates the plant cell more slowly from solutions of its salts than from the solutions of free bases, and still more slowly from solutions of the salt to which acid has been added.

The relative activity of the free base and of the salts of cocaine and its derivatives in blocking nerve was determined by GROS (1910). He found the free base to be much more efficient. SYMES and VELEY (1911) were unable to confirm these results when using solutions of the pure alkaloid base and of the salt. CRANE (1921) used cultures of *Paramoecium* in solutions having pH values from 9.6 to 5.0 of a number of alkaloids and found that there was a greater toxicity in the action of the drug when the concentration of undissociated base was increased.

HENRY and BROWN (1923) showed the influence of toxicity of quinine, emetine and conessine upon protozoa in hay infusion. The higher the pH, the greater the toxicity.

POIJÄRVI (1928) studied the rate of penetration of weak bases including cocaine, atropin, piperidine and brucin into *Rhoeo* and *Tradescantia*, noting the change in color of anthocyan and in roots of *Lemna* intravitaly stained with neutral red. He concluded that the rate of entry of weak bases of equal concentration of molecules obeys Fick's Law.

BORESCH (1919) found that addition of OH ions increases the ability of alkaloids to become emulsified, whereas, the addition of H ions decreases it. This changed the dissociation constant of the substance. Many others have recognized the importance of pH upon the effects produced. Among these may be mentioned GOLYAKHOVSKII 1934, PROWAZEK 1910, IRWIN 1928.

THIEULIN (1920) using collodion membranes impregnated with castor oil or lecithin found that the least dissociated salts diffused the best.

Permeability to dissolved gases. To give a complete picture of agents affecting permeability the dissolved gases normally present in and around living cells should be mentioned.

These include oxygen, nitrogen, carbon dioxide and ammonia, and in some cases, hydrogen, methane and hydrogen sulphide. The primary interest in experiments with oxygen, nitrogen and carbon dioxide lies in their relation with respiration and oxidations, rather than with permeability alone, since living tissue is presumably freely diffusible to these gases. In addition might be included CO and methylamine.

Table XXII. Permeability Constants for Oxygen

Organism	Tissue or Cells	Gas	t°C	Permeability Constant, P	Observer
Arbacia .	Fertilized Eggs	O ₂	—	cm ² /sec $\times 10^{-7}$ 71	RASHEVSKY (1933)
Sheep .	Red Blood Cell	O ₂	37	M/cm ² /sec/M/L $\times 10^{-7}$ 17	HARTRIDGE and ROUGHTON (1927)

Quantitative measurements from the permeability point of view have been made by WRIGHT (1934) and WILSON and WRIGHT (1934) on the gaseous interchanges through visceral pleura and tissues with CO₂ and O₂. Permeability constants ($P \times 10^{-7}$) for CO₂ are given in Table XXI for weak electrolytes.

Hydrogen does not diffuse from the pleura space into the lungs and vice versa, according to BESTA (1934).

The rate of distribution of dissolved gases between red blood cells and their fluid environment has been investigated by HARTRIDGE (1927) for O₂ (See Table XXII); by DIRKEN and MOOK (1931) for CO₂ (Table XXI); by RASHEVSKY (1933) for *Arbacia* eggs (Table XXII) in which the permeability of the cell surface for O₂ was calculated from oxygen pressure/oxygen consumption curves. Agreement with the work of GERARD on sea urchin eggs when two limiting factors are considered, such as the diffusion factor, D , and the permeability factor h , was found, but in the case of other cells, no agreement was noted.

These are merely a few examples of the vast literature concerning the important studies on such gases as O_2 , CO_2 , N, and CO. The process of photosynthesis deals with these, the whole subject of respiration, plant and animal, and oxidations and reductions. They are concerned with molecular diffusion and diffusion processes in living and non-living systems, and the reader is referred to such articles as that by JACOBS (1931) with reference to living systems, and to CADY and WILLIAMS (1934) for non-living systems.

CHAPTER XI

PERMEABILITY TO STRONG ELECTROLYTES

The experiments which have been done on the permeability of living cells to electrolytes comprise volumes. Among the reviews which have been compiled under this title, those of HÖBER (1926), GELLHORN (1927, 1933) and JACOBS (1935) may be mentioned. This subject includes also those important subheadings as antagonism, the electrokinetic phenomena (see FREUNDLICH 1932), electrical impedance, such as the experiments of K. S. COLE (1937) on *Arbacia*, BLINKS and OSTERHOUT and colleagues on electrical potentials of *Valonia*.

Salts of the strong electrolytes are found inside of all cells and can be made to enter experimentally. Salts are absorbed from their environment, which is either natural waters or internal fluids. There is a similarity between sea water and serum which has led some to the idea that body fluids represent roughly the composition of Ordovician or Silurian sea water (MACCALLUM 1926). This idea is disputed by H. W. SMITH (1932). (See also BALDWIN 1937).

The following table shows this similarity:

Table XXIII

Medium	Na	K	Mg	Ca	Cl	SO ₄
Sea Water480	.010	.054	.011	.557	.028
Serum137	.006	.011	.021	.109	—

See WHEELER (1910) and ABDERHALDEN (1914).

Cells may be "naked", i. e., without cell wall, like amebas; they may be surrounded by cell walls, as in the case of most plant cells or by cutin as in some invertebrates. Cells may have

"adsorption films" adhering as discussed by JENNY and OVER-STREET (1938). These are factors which should be taken into consideration in the study of the permeability to salts.

Within cells of plants it is possible to obtain in some cases pure sap. Two analyses are of interest here, the first showing sap much like sea water, which is the surrounding medium in both cases, and the second showing marked selective accumulation of K (BROOKS 1930, and ZSCHEILE 1930).

Table XXIV

Species	Na	K	Mg	Ca	Cl	SO ₄
<i>Halicystis osterhoutii</i>557	.006	.017	.008	.603	trace
<i>Valonia ventricosa</i>001	.610	.001	.002	.620	"

See BLINKS (1930) and BROOKS (1933).

The high ratio of K/Na is also seen in the sap of various species of Characeae (See COLLANDER 1936).

Most other analyses of marine material refer to extracts of whole tissue (BERTRAND, et al. 1927, 1928). For example, the ratio of K to Na in certain sea weeds was as follows: *Fucus serratus* .85; *Rhodymenia palmata* 77.8. There is also variation with seasons in individual species (CAMLONG and GENEVOIS 1930).

Among fresh water plants the best analyses refer to saps of different species of *Nitella* and *Chara* (HOAGLAND and DAVIS 1929). The degree of selective absorption indicated by the ratio K/Na for *Nitella* was found to be .51—1.48 in the sap as compared with .42 in the surrounding pond water. The ratio for a species of *Chara* from brackish water is intermediate between *Nitella* and *Valonia* (COLLANDER 1930).

Vascular plants show a somewhat similar range of variation (BERTRAND and PERIETZEANU 1927). K/Na ranges from 1.15 for the marine *Zostera marina* through *Malva rotundifolia* 3.06; *Zea mays* 48.2; to *Sambucus nigra* leaves 1040. For various halophytes see ZELLNER (1927). ANDRE and DEMOUSSY (1925) have pointed out that in sugar beets K/Na is higher the younger the root and the further from the vessels the sample is taken. This is suggestively related to the higher diffusion velocity of K.

Animal cells, not being very large nor having large vacuoles, have so far been studied only as whole tissues. Variations similar to those found in plants occur. Ratios of K as compared with Na content of muscle tissues are given in a review by FENN (1934, 1936) showing the high concentration of K and changes produced in this ratio by stimulation. The loss of K from muscle during activity has not been satisfactorily explained. FENN suggests that the muscle fiber may be surrounded by a K-permeable membrane. This is taken up later on under "Theory of Ionic Exchange".

Table XXV. Average Electrolyte Changes in Rat and Cat Gastrocnemius Muscles during Stimulation and Recovery

	Rat			Cat		
	Average content	Change in stimulation	Change in recovery	Average content	Change in stimulation	Change in recovery
K . . .	47.3	— 6.1	+ 3.6	45.8	— 5.3	+ 1.8
Na . . .	7.6	+ 8.3	— 6.4	5.7	+ 8.7	— 1.9
Cl . . .	5.4	+ 2.8	— 2.0	9.0	+ 4.1	— 1.2
H ₂ O . .	318	+49	—43	301	+76	—38

Water is expressed in cc. per 100 grams dry weight. All other figures are in millimols per 100 grams of dry weight. Changes in cats during recovery are based on seven pairs of analysis of tibialis and gastrocnemius muscles in only two cats. One gastrocnemius muscle showed no recovery of potassium.

The same high K content is found in nerves (See FENN, COBB, HEGNAUER and MARSH 1934) and changes in relative proportions of cations observed when nerves were placed in different pH solutions, different concentrations of electrolytes or non-electrolytes. The K shift of frog sartorius muscle found by FENN and COBB (1935), in which K moves into a muscle under increased CO₂ tension, is explained by them on the basis that muscle is K-permeable and Na- and anion-impermeable; or that K is immobilized in the tissue because it is combined with indiffusible anions. Muscle is difficult to treat since some of the Na may be found on the surface of the fibers (MOND and NETTER 1932).

Erythrocytes (ABDERHALDEN 1914) show a high ratio of K/Na in pig, human, horse, for example 0.106/tr., and a low ratio

in the case of ox, cat, dog, for example 0.015/0.079. Egg cells have been exhaustively studied by BIALASZEWICZ (1929). He endeavours to determine K and Na as they exist free in the dispersion medium of the cells. K/Na ranges from 1.35 in *Arbacia pustulosa*, where some sea water contaminating the sample may have unduly lowered the figure, to 136 in *Labrax lupus*, a teleost fish. No mammalian eggs were analyzed. INGRAHAM and VISSCHER (1933) found that the potassium concentration is invariably greater in gastric juice of dogs as compared with plasma, sometimes as great as fourfold, while the Na concentration is lower than in plasma. They suggest that the difference is due to the entrance of potassium into the lumen of the gland from the secreting cells while sodium enters through the intercellular spaces. This relation between "inside" and "outside" has been suggested by several, including FENN, to include high K content within the cell and high sodium in the spaces. However, it would not fit in the case of *Valonia*, where there occurs high K in the vacuole, if vacuole can be considered as "space". In the case of the marine green alga, *Codium Bursa*, it was found that K is present in the central cavity in higher concentrations than in the surrounding sea water. In this plant the central cavity is only partially separated from the surrounding sea water by living protoplasm (S. C. BROOKS 1933).

There appears to be a general correlation between the degree of selective accumulation and the "activity" of cells as judged by rate of growth, secretion, metabolism, etc. Embryo tissues, which grow rapidly (MANKIN 1930), have a high Na and K and low Ca. In chick embryos at the 13th day Ca rises, and other cations and H₂O fall, approaching the proportions found in mature animals.

Mg/Ca shows similar progressive change with age (DELBET and BRETEAU 1930).

Table XXVI

Tissues	Mg/Ca
Infant testicle	1.0
Adult ,, 8
Senile ,, 34
45-year-brain8
68- ,, ,, (male)36
65- ,, ,, (female)33

Similar correlation with growth rate occurs in regeneration and healing in rabbits (BRICKER and LAZARIS 1931), and regenerating limb buds of amphibia (THOMAS and KOSTAREFF 1927).

Hibernation, which is general inactivity of the whole animal, is also characterized by low ratio K/Ca; normal ratio in blood of marmots being 3.47/4.33; in hibernation, 0.95/2.57 (FEINSCHMIDT and FERDMANN 1932).

Neoplasms show some of the most interesting relations. Plant galls produced by insects showed the following ratios to the corresponding healthy shoots of leaves in their content of the elements indicated (BRANHOFFER and ZELLNER (1920), see Table XXVII):

Table XXVII

Plant	Na	K	Ca	Si	Cl	CO ₂
Elm	"zero"	2.22	.53	.34	.96	12.48
Beech	"zero"	1.62	.59	.16	2.14	1.62
Fir	"tr"	1.67	.21	.12	2.26	1.78

(After BRANHOFFER and ZELLNER 1920.)

Animal neoplasms show similar relations (BEEBE 1904). Correlation between malignancy and mineral content was measured by CLOWES and FRISBIE (1905) as follows:

Table XXVIII

Time Inoculation to death	% of total: Na, K, Ca		
	Na	K	Ca
25 days	75	19.5	5.5
38 „	—	13.6	—
44 „	85.8	6.5	7.6
48 „	90.8	0	9.2

These change are characteristic of the tissues, but apparently not of the body fluids, e. g., serum (PITTS and JOHNSON 1930). See also data by WATERMAN (1922) and WOLF (1923), and microincineration data of SCOTT and HORNING (1932) and OLCH (1933).

The penetration of cations and anions has been especially studied in relation to red blood cells. Species differences are observed. The cells of human, hog, horse and rodents, for example, contain K but relatively little Na; whereas, those of cow, sheep, goat, dog and cat contain Na and relatively little K. See JACOBS (1931) and PONDER (1934).

HÖBER (1936) has studied the penetration of some organic anions, i. e., aliphatic fatty acids, aromatic sulfonic acids, aromatic carbonic acids, and aliphatic oxy-, di- and tricarbonic acids, into red blood cells. He concluded that the anion permeability of the red cells is in many respects consistent with the permeability of the selective anion-permeable collodion membrane, but that the presence of lipoids in the cell surface co-operates as a strong factor, especially favoring the entrance of the anions of the fatty acids and of the aromatic carbonic acids. In the cells of sheep and ox there appears to be a greater relative surface area occupied by lipoids than in the cells of man, rat and mouse.

Characteristic differences in the time of hemolysis by various agents show differences in the erythrocyte of albino rat and albino mouse (JACOBS, GLASSMAN and PARPART 1938). The retarding effect of dilute solutions of electrolytes upon the hemolysis of red blood cells, increases rapidly with the valence of the cations present. The valence of the anions is much less important, but if anything, acts in the opposite sense. Under certain conditions, ionic forces may modify the rate of osmotic intake of water by the erythrocyte (JACOBS and PARPART 1932).

The effect of cations, antagonism of ions and the absence of cations on membrane formation of echinoid eggs has been shown by MOORE (1930, 1935), BRADWAY (1936), FUKUDA (1935).

Anions have been studied by H. W. SMITH (1925, 1926) in connection with cell division of the eggs of *Echinarachnius parma* and with contraction of heart muscle. He concluded that the action of carbonic, acetic, propionic and other weak acids depended upon their properties as acids rather than on any specific physical or molecular action of the anions. COLLETT (1921) also found no evidence for toxicity to *Paramoecium* or *Euplotes* on the part of anion or molecule of lactic, succinic or tartaric acids in low concentrations. W. H. COLE (1932) studied the effects of a series of normal aliphatic acids on the stimulation of the rock bar-

nacle, *Balanus balanoides*, using the time of closing of the valve as a criterion. He concluded that stimulation is correlated with the potential of the anion of the acid and with the concentration of that ion near or at the receptor surface as determined by the length of the carbon chain.

The ciliated cells of *Mytilus edulis* were found to be insensitive to the following anions: Cl, NO₃, Br, I, acetate and SO₄ (GRAY 1922). PUMPHREY (1932), using the inner epidermis of onion scale, showed a resemblance to the dried collodion membrane in being very impermeable to anions.

Theories of ion accumulation. The theory involving the penetration of electrolytes has been a controversial subject. (See current reviews on selective accumulation of electrolytes as given by HOAGLAND (1933, 1937), STEWARD and MARTIN (1937), OSTERHOUT (1936), S. C. BROOKS (1938).) In the case of non-electrolytes, it is generally agreed that the molecule diffuses readily into the cell. Strong electrolytes are not accumulated in the same way as non-electrolytes. Most dilute solutions, such as those which are found in relation to plants and animals are, according to the DEBYE-HÜCKEL theory, completely dissociated with absence of ion pairs. (See also VAN RYSELBERGHE 1933). However, sea water and serum, with concentrations of ions around 0.1 M do not fall under the category of "ideal dilute", and according to NERNST (1928), probably contain ion pairs. Ion pairs are equivalent to molecules, i. e., they have no net charge. In the case of physiological fluids, one might assume that most of the salts of the strong electrolytes are "practically" completely dissociated, but contain some "ion pairs".

Accumulation by molecular diffusion. The question arises as to how these strong electrolytes pass through the plasma membrane, as ions or as molecules. OSTERHOUT (OSTERHOUT 1932, 1933, 1936; OSTERHOUT and KAMERLING 1935; JACQUES and OSTERHOUT 1932) has attempted to explain the accumulation of ions in the cell on the basis of entrance of ion pairs. In the large cell, *Valonia*, the concentration of KCl in the sap greatly exceeds the concentration in sea water (i. e., ion activity product). In some species KCl is over 99% of the total salt content of the sap. OSTERHOUT believes that K enters as KOH or "KG" ("G" being guaiacolate plus p-cresol in an analogous artificial cell). In order to prove his assumption, models and mathematical for-

mulations have been proposed and comparisons made with results found with living cells. His earlier theory (OSTERHOUT 1928) assumed that in *Valonia*, where the preponderance of salt in the sap is KCl, K and Cl combined momentarily at the surface of the plasma membrane and penetrated as a molecule. His later interpretation is that K combines with OH and penetrates as a molecule in the form of KOH¹); that KOH combines at the outer surface of the plasma membrane with an organic acid, HC, to produce KX and water. At the inner surface another reaction takes place in which KX unites with H₂CO₃ and forms organic acid again. In this way, the outer layer loses a K-ion and the inner layer an H ion, and thus the neutrality of the sap is accounted for. However, we may note that the validity of this hypothesis depends on the existence of an outward gradient of H₂CO₃ or its ions. This is not always the case in the experimental data.

Selective accumulation of ions implies that these ions are found free in cell fluids or cytoplasm in concentrations, or better, activities, different from those in the external medium. Former experiments, in which the cell-sap was found to contain ions in a greater concentration than was found in the external solution, have implied that this accumulation occurs against the activity gradient. The later experiments in which the relations between protoplasm and external solution have been studied (BROOKS 1937, 1938) show that the seat of accumulation is really in the *protoplasm* rather than the *sap*, and that other factors can be employed to help in explaining this accumulation. The attempt to formulate a physico-chemical explanation of such phenomena has led to hypotheses which link together oxidation, electromotive forces and growth.

The fact that accumulation is most conspicuous in the case of ions and occurs rarely if at all in the case of molecules, suggests that electromotive forces serve to move ions into the cell. The potential differences actually observed between the protoplasm or the sap of living cells and the solutions bathing them,

¹) After assuming that K enters as KOH, OSTERHOUT then states that "protoplasm is normally very permeable to KOH". This is contrary to the evidence of all experiments in which KOH has been used. (See HARVEY 1914; M. M. BROOKS 1923; and other experiments in which cells were placed in solutions of KOH without producing any change in pH as indicated by color inside.)

often oppose the entrance of certain ions which are conspicuously accumulated. UMRATH (1938) has found that the protoplasm of *Valonia macrophysa* is more negative than either the external solution bathing it on one hand or the sap bathing it on the other. (See also BROOKS and GELFAN 1928.) No one electromotive force can account for the simultaneous accumulation of both anions and cations which often occurs. This leaves two alternatives: either accumulation is not due to e. m. f.'s; or the experimentally observed e. m. f.'s are not those causing accumulation.

OSTERHOUT's theory is based on the former alternative. His artificial models will cause K to pass from a dilute alkaline K-guaiacolate solution through a layer of guaiacolic acid into a more concentrated KHCO_3 solution saturated with CO_2 . Since the latter solution is at a more positive potential, the K, if ionized, would be moving against the e. m. f. in the system. What happens is that the K-guaiacol is decomposed at the guaiacol- KHCO_3 interface by the H_2CO_3 (which is constantly renewed) and hence its concentration kept lower at that interface than at the other interface of the guaiacol layer. Hence molecules of K-guaiacolate diffuse steadily to the H_2CO_3 interface, there forming KHCO_3 and guaiacolic acid. The KHCO_3 remains in the aqueous solution in the form of ions while the guaiacolic acid diffuses back to the other interface. While this model simulates cation accumulation, as illustrated by ratios of KCl in the sea water as compared with that in the sap of *Valonia*, OSTERHOUT does not offer a mechanism for the simultaneous accumulation of anions and cations (which is a requisite for living cells), and states, "The cell apparently has a device for making Cl^- move out more slowly than it moves in" (OSTERHOUT 1936). See also TEORELL's (1937) models on ionic distribution in a "steady state" type of system.

Accumulation by ion exchange

The theory which has been offered by BROOKS (S. C. 1929, 1935, 1937, 1938) for selective accumulation of strong electrolytes depends upon several factors, including (1) non-equilibrium states in living cells; (2) the influence of metabolism upon concentration; and (3) difference in rate of diffusion of ions as first observed by ANDRÉ and DEMOUSSY (1925). They explained the abundance of K as compared with Na in sugar beets on the basis of mobility of ions. In addition to this (4) the membrane may be thought

of as composed of paths which are in some places anion-permeable and in others cation-permeable, resembling a mosaic in structure. (See HÖBER and HÖBER 1928.) These factors will be discussed at greater length.

Non-equilibrium and metabolism. Since the cell is constantly growing while alive it never attains equilibrium. This state has been termed "dynamic equilibrium". (See A. V. HILL 1930.) The continuous metabolism affords a constant source of organic ions and only when the process stops is there a return to equilibrium between the outside solution and the concentration inside the cell. The relation between high metabolic activity and accumulation of ions has been demonstrated experimentally by the work, for example, of HOAGLAND and DAVIS (1929) in which bromine ion is accumulated to a marked degree by *Elodea* in concentrations greatly in excess of those in the external solution. The relation between ion uptake and glycolysis has been shown in tumor tissues which are rich in K ion and produce lactic acid. These metabolic processes then furnish by dissociation the ions in the cell and furnish the energy which is needed for ion exchange. We may suggest the types of ions which might be active in ion exchange by mentioning ammonia, $(\text{CH}_3)_4\text{NOH}$, guanidines, betaines, purines and pyrimidines, histamine, creatinine, etc. among substances furnishing organic cations; and bicarbonates, lactates, pyruvates, succinates and malates as anions.

Protoplasm is the active seat of accumulation, and also the seat of metabolism, (notably respiration, oxidation and reduction, and photosynthesis)¹⁾ whereby ions are produced. The protoplasm is comprised of proteins which are large molecules with many acidic and basic groups dissociating as polyvalent ions. In the synthesis or breakdown of proteins smaller molecules arise, i. e., amino-acids and the smaller peptides. These ions are available for exchange and tend to diffuse along their diffusion gradients. In the case of vacuolated cells, these ions diffuse outwards into the external solution and inwards into the sap.

The rate of metabolism whereby organic acids and bases are produced will affect the pH and the dissociation of the proteins

¹⁾ This scheme of photosynthesis proposed by FRANK and HERZFELD (1937) suggests that this process produces weak acids. In green cells photosynthesis may furnish counter anions and these will promote anion intake.

of which the protoplasm is composed. Therefore the concentration of cations and anions will partly depend upon the pH.

The interior of the actively metabolizing cell contains a concentration of diffusible ions greater than that in the exterior. These ions will therefore move outward through the mosaic surface, both to the external solution and to the sap. Since no oppositely charged ion can accompany an ion through any single selectively permeable area, the electrical neutrality will be maintained by counter diffusion of other similarly charged ions in amounts electrically equivalent to the ion leaving the cell.

The accumulation of inorganic ions requiring movement of ions against their activity gradients demands the following essentials, irrespective of the mechanism of ion intake postulated. This presumes that the mechanism is adequate, a stipulation not fulfilled by membrane equilibria (DONNAN ref.) or various diffusion theories (TEORELL, OSTERHOUT, but see CASSEL and MILES 1939). Both the OSTERHOUT "molecular" and the BROOKS "ion exchange" hypotheses require an ultimate ion exchange, so that organic or other ions must be supplied by the protoplasm (not the sap) which are replaced by incoming inorganic ions.

It has been shown by STEWARD, BERRY and BROYER (1936) that in carrot and artichoke storage tissues, the accumulation of K and Br depends upon the oxygen concentration. This agrees with the work of HOAGLAND and BROYER (1936) in which young excised barley root systems were immersed in a solution of potassium salt contained in bottles with a stream of oxygen or nitrogen gas passed through the solution. The aerated roots supplied with oxygen quickly built up high salt concentration, while those supplied with N_2 accumulated little or no salt from the external solution. However, it has been pointed out by WHITE (1937) and by ROBBINS (1939) that excised roots of tomato and pea plants lack the property of synthesizing the growth-promoting substance, thiamine, or vitamin B_1 , and that they obtain their supply only from the shoot. If this principle applies also to barley plants and similar material, such as the excised root systems of the work of HOAGLAND and BROYER (1936) and PREVOT and STEWARD (1936), this material cannot be used in place of normal roots to show normal effects on metabolism and growth.

ROSENFELS (1935) showed that an induced rise or fall in the rate of respiration is closely but not exactly paralleled by a similar

rise or fall in the rate of bromide absorption of whole *Elodea* plants.

Since oxygen requirements vary greatly with the kind of plant used, it is not necessarily a general conclusion that in every case oxygen is necessary. The dependence of salt accumulation upon aerobic respiration is only of specific application. Besides deriving energy from aerobic respiration, it can also be derived from aerobic glycolysis, which does not give CO_2 as an end product or from anaerobic glycolysis, when no O_2 is required and whereby lactic acid or some other organic acid or alcohol is produced in place of CO_2 . Therefore, those experiments, which give as their basis for theories on ion exchange the relation between the products of aerobic respiration and ion intake, are correct only insofar as they apply to the special cases.

STEWART and MARTIN (1937) have attempted to measure the metabolism of *Valonia ventricosa* in experiments at Tortugas and have failed to find any appreciable production of CO_2 . They conclude, therefore, that *Valonia* has a low rate of metabolism, and the results with these plants should therefore not be used for experiments having general application. It seems to the writers that these conclusions are not justified for the following reasons: The method of taking measurements on CO_2 production was unsatisfactory, as recognized also by the authors. No manometric readings were done. The method for measuring CO_2 was based upon titration of CO_2 obtained in absorption towers connected with sea water containing either *Valonia* or no *Valonia* plants. 100 grams of plants were used. No measurable amount of CO_2 was obtained. This is compared with measurable CO_2 when 100 grams of *Ulva* was used.

The comments on this method are as follows: If one estimates approximately the weight of the protoplasm contained in 100 grams of *Valonia* plants as compared with that in *Ulva*, there appears a considerable discrepancy. For example, estimating that the average of these *Valonia* cells was 1 cm. in diameter, and the thickness of the protoplasm $10\ \mu$, then the weight of the protoplasm of each plant would be approximately .03 gm. (Estimated on the basis of the weight of water as 1 gm. per cc.) On the other hand, the volume of a sphere 1 cm. in diameter would be approximately 0.5 cc. In other words, the weight of the protoplasm, the metabolizing part of the plant, would be only about 6/100ths

of the amount of protoplasm taking part in a plant like *Ulva*, which has no large vacuole. HOFFMANN (1929) estimated that *Ulva* produces 1.2×10^{-4} gm. CO_2 per gram fresh weight of plant. For *Valonia*, even at the same rate, this amount would approximate 7.2×10^{-6} gm. of CO_2 . To measure these small quantities, it would appear that more refined methods were needed. Therefore, these experiments could not be used to prove anything.

On the contrary, it has already been found by M. M. BROOKS (1923) that the sap of *Valonia ventricosa* always contained a considerable concentration of CO_2 . This could only have been produced by the metabolism of the plant. The method used was by pH determinations. Before aeration, the expressed sap had a pH of 6.0 to 6.4 and after aeration, a pH of 7.0, as determined by indicator methods. While this is a relatively crude method, nevertheless, it shows a definite and measurable amount of CO_2 present. In a more refined experiment, (M. M. BROOKS 1930) the glass electrode was used to measure H-ion concentration in the sap which was quickly and carefully expressed immediately before measuring, and the pH was found to be 6.0. Since the sap contains mostly nothing but KCl, such difficulties presented by buffer action, for example, as are found with salts of the sea water are avoided. The fact that there is a measurable concentration of CO_2 at any time in the sap argues in favor of a measurable rate of metabolism.

In addition to aerobic respiration which is carried on by *Valonia*, there may be some glycolysis as evidenced by the presence in the sap of small amounts of organic matter. (See OSTERHOUT 1922, M. M. BROOKS 1925.) Therefore, contrary to STEWARD's thesis that *Valonia* has a low metabolic rate, it should rather be considered as having relatively a high metabolic rate. The positive evidence of a high concentration of KCl in the sap should rather argue in favor of this conclusion. *Valonia* evidently grows at a rate which keeps the concentration of KCl at a steady level for a considerable time. As K ions enter the osmotic pressure is increased, thereby attracting more water which again keeps the KCl at a constant dilution, so that the ultimate appearance is that of no further intake of KCl. As the cells become older, (larger) they gradually become more alkaline (See M. M. BROOKS 1923) due probably to the decrease in rate of metabolism, increase in permeability and intake of sea water. See also WEBER (1931)

who showed that younger cells of *Spirogyra* are impermeable to urea but become permeable as the cells age. Cancer cells show a positive correlation between high concentrations of K and a high rate of metabolism, but they have a low aerobic respiration, obtaining their energy rather from glycolysis (WARBURG 1925). Red mammalian blood cells, as well as cancer tissue have high aerobic glycolysis. See BARRON (1929), HARROP and BARRON (1928), and WARBURG (1925). Yeast cells grow and divide in an anaerobic environment.

Even where respiration is the primary source of metabolism, nevertheless other processes serving as sources of energy, as for example, glycolysis, no doubt also proceed simultaneously. So that discrepancies in the intake of ions, as compared with concentrations of CO_2 measured, are not the complete picture, because anions and cations produced by such other sources should also be considered.

The relation between the pH of the sap of *Valonia* and that of the sea water in which the plant lives has been used by OSTERHOUT and others in theories on penetration. This difference amounts to 2.5 pH units. However, HOAGLAND has shown in experiments with barley roots placed in solutions having a pH as low as 4.0, in which the H-ion concentration is greater outside than inside, that accumulation of K and Br occur just the same. From these experiments it would appear that the K ion would not exchange for the H ion because the gradient of H ions is in the wrong direction, assuming that the *protoplasm* is being used for comparison rather than the *sap*, and that the pH of the protoplasm must be around neutrality. Therefore the exchange of K ions must be with some other cations rather than H exclusively.

When the H-ion concentration outside is less than that inside, there may be an exchange of K^+ for H^+ . This was the case assumed by S. C. BROOKS (1937). Actually, other cations besides H are no doubt responsible for exchanging with K-ions, as suggested by S. C. BROOKS (1938).

In some cases, the CO_2 produced by active cells is many times the chemical equivalent of the salt absorbed (STEWART 1933). STEWART (1937) states that the effect of salt concentration upon absorption is relatively far greater than upon respiration. This may be true for very dilute concentrations of salt of the order of .00075 M which produces very little effect upon respi-

ration. In higher concentrations of salts however, as for example, 0.03 M, there are definite increases in the rate of CO_2 production by such organisms as *B. subtilis* as shown by M. M. BROOKS (1919, 1920) in which an increased rate of around 100 percent was produced. The final word in the relation between salt concentration and rate of respiration has not been given.

The conclusion of STEWARD, BERRY and BROYER (1936)¹⁾ that *Valonia* has ceased growth and active metabolism because they were unable to find a high rate of aerobic respiration, or that any cell which has a high "respiration" in nitrogen should be included under this heading does not appear general.

HOAGLAND (1923, 1929) and HOAGLAND and HIBBARD (1926) have shown that the accumulation of electrolytes by some plants such as *Nitella* and *Elodea* is increased by light. *Nitella* cells can accumulate both K and Br under conditions of continuous illumination, while the CO_2 is being removed from the system.

Little is known concerning the equilibrium between CO_2 production in *Valonia* and its uptake by the process of photosynthesis. This may be so nicely balanced that little residual CO_2 remains to be measured. In the case of potato and storage tissue and roots, since no chlorophyll is present, the complications of photosynthesis are avoided. For details concerning salt accumulation by higher plants see review by HOAGLAND (1937).

In the case of animal cells certain agents have been used to inhibit metabolism and the effect upon permeability under various criteria noted. These agents include usually KCN, which inhibits oxidations, and CH_2ICOOH , which inhibits glycolysis. The experiments of HÖBER and FERRARI (1933) show that the stellate cells of frog liver and the leucocytes are insensitive to cyanide but that the formation of lactic acid by mono-iodo-acetic acid is interrupted; that the secretion of glomerular urine in frog kidney, as well as the resorptive function of the tubules for Cl and the secretory function towards phenol red is reversibly affected by both of these reagents. (See also PREVOT and STEWART 1936.)

Mobility of ions. Mobility is an important factor in the study of diffusion of ions. The series $\text{Li} > \text{Na} > \text{K} > \text{Rb} > \text{Cs}$ generally follows for rates of free diffusion. The series of univalent alkali earths beginning with the physiologically im-

¹⁾ Page 364.

portant Na which has electron shells of the structure (2) (8) (1), and on up to Cs which begins the third long period with six shells of the structure (2) (8) (18) (18) (8) (1), serve to illustrate the effect of these additional shells upon their penetration through cells. As the number of shells increases, the positive charge is farther and farther removed from the outside orb of electrons which rotate about it, and for this reason the water molecules which are dragged along with these ions are more loosely bound than in the case of Na, for example, which has only 3 shells. Therefore in the case of Rb, which has 5 shells, the water molecules can be pushed off as the Rb ion goes through the plasma membrane, so that the diameter of the Rb ion is actually smaller than that of the Na ion with its adhering water molecules. In the same way K^+ will supersede Na^+ .

The accumulation of K by plants is almost a universal phenomenon. Attempts were made by S. C. BROOKS (1932, 1933, 1938) to see whether the same law holds good in the penetration through *Valonia* of these ions in accordance with the above series. In comparing results on Rb with those on K he found that Rb was accumulated at a faster rate than K under similar conditions of experiment.

Since the mobility of K^+ is greater than that of the other ions, K^+ will among common cations enter most rapidly, other factors being equal, so that at ultimate static equilibrium the following relations obtain:

$$\frac{(H^+)_{\text{i}}}{(H^+)_{\text{o}}} = \frac{(Na^+)_{\text{i}}}{(Na^+)_{\text{o}}} = \frac{(K^+)_{\text{i}}}{(K^+)_{\text{o}}} \text{ or } \frac{(K^+)_{\text{i}}}{(K^+)_{\text{o}}} = \frac{(K^*)_{\text{i}}}{(K^*)_{\text{o}}}$$

We can thus account for the accumulation of any ion in the sap of a living cell up to a concentration at which the ratio of its activity in the surrounding solution is equal to the same ratio, for example, of hydrogen ions inside and outside. (H ions are used as a basis of comparison). In this process of ion exchange, the concentration of each cation taken in will be proportional to the product of two factors: the activity gradient for that ion in the direction of entry into the cell, and its mobility through the plasma membrane or the protoplasm. Although there is less potassium than sodium in sea water, the superior mobility of the K ion would easily account for the fact that more potassium than sodium is exchanged for hydrogen ions. In the sap of *Valonia*,

there is an absence of bivalent ions in uninjured plants. This may be explained by the low penetrability together with their low activities in sea water. The greater mobility of I ion may account for its accumulation in many cells.

HÖBER and HÖBER (1928) showed the rapid exchange of Br for Cl in cell sap of *Valonia utricularis*, and absence of exchange when the cells were placed in isotonic glucose solution.

The experiments of GRAY (1922) on the rate of ciliary movements as influenced by ions is significant. He showed that movement is slowest in solutions of Li and fastest in solutions of K at the same H-ion concentration. This may be due to removal of nitrogenous wastes by ion exchange for K^+ .

The alleged exception in the case of different erythrocytes where some species contain a preponderance of Na and others K is not explained. Apparently other factors as yet not proven must be considered. They have different rates of respiration and glycolysis depending upon the species. The relation between these facts and their permeability to Na or K is not well understood. It has been suggested that they were permeable during formation in the hematopoietic tissue. In the course of their development their permeability to Na or K decreased so that in their mature state they appear to be impermeable to cations in general.

Sap versus protoplasm. It seems important in this connection to emphasize the fact that in dealing with experiments on *Nitella* and *Valonia*, the analyses which have been made of the external solution, the sap (and lately also the protoplasm) are not exactly comparable with those made on plants like *Elodea*, barley shoots, potato tissues and other cells of which the sap cannot be extracted. In the latter case, analyses consist of the whole tissue.

In the experiments of HOAGLAND and colleagues on the accumulation of Br ion into *Elodea*, (in contrast to *Nitella*) the whole plant was used for analysis as the cells of this plant are not amenable to separation into protoplasm, wall and sap as in the case of *Nitella*. Therefore, the observed accumulation may have taken place chiefly in the protoplasm.

Chara and *Nitella* can accumulate in their vacuolar sap the principal ions from the external solution, in concentrations exceeding those obtained outside the cell. *Valonia* accumulates only KCl primarily. The vacuoles of *Chara* and *Nitella* appear

to be smaller in comparison to the protoplasm than in the case of *Valonia*. In expressing the sap of *Nitella*, it has always appeared to the authors that some of the constituents of the protoplasm can easily be included in the process of extraction unless particular care is taken to avoid this.

In the earlier experiments with *Nitella* and *Valonia*, in which comparisons between external solution and "inside" (referring to sap) are made, it has been found that the sap in many cases contains greater concentrations of certain electrolytes than are found in the external solution. A great deal was written about the differences between the concentration of electrolytes "outside" and "inside" and no satisfactory solution was known. Since the concentration "outside" has been found to be lower than that in the sap, it has been maintained that accumulation of electrolytes takes place "against the concentration gradient".

It has been shown by S. C. BROOKS (1933) in experiments with *Valonia* that the real seat of concentration of these electrolytes is the *protoplasm* rather than the sap; that the concentration in the sap is more dilute than that in the protoplasm, and that therefore the diffusion from the protoplasm into the sap is not against the gradient but with it.

To show directly the role of *protoplasm* in the process of accumulation, experiments were devised by S. C. BROOKS (1937, 1938) with *Nitella*, and separate analyses of protoplasm, sap and wall were made. For this purpose radioactive isotopes, H^*Cl , Rb^*Cl , Na^*Cl and KBr^* were used and estimations were obtained instantly by measuring the number of electron impacts for each separate part of the plant by means of a GEIGER-MÜLLER (β -ray) counter. It was found that the protoplasm accumulates to an enormous degree radioactive K^* , for example, before it is passed on into the sap. The wall is not a factor in accumulation. The sap contains less of the entering ion, but this concentration increases with time. Diffusion from protoplasm into sap is *with* the concentration gradient rather than *against* it.

The concept which we wish to bring out is that the protoplasm is the agent which is important in accumulating electrolytes; that the concentration of these electrolytes in the protoplasm when experimentally produced can be quantitatively measured minute by minute and their course followed through the cell. The question arises then, why is there an accumulation in the protoplasm?

The mosaic nature of the plasma membrane or the superficial layer of the living cell is a natural concept. This region seems to consist of a relatively solid protein gel, or at least to contain such material. It seems very doubtful whether the "lipoid layer" is essential for all cells as indicated by consideration

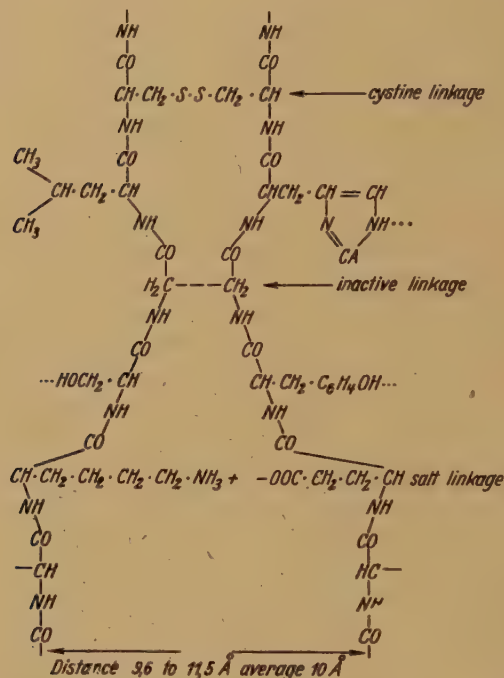


Figure 13. Diagrammatic representation of the side-chain or grid linkages between parallel polypeptide chains of β -keratin. After Block in Schmidt' Chemistry of the Amino Acids and Proteins.

of experiments on their permeability and other experiments (BROOKS 1937). Its presence in such cells as neurons (SCHMIDT 1937, 1938) and the relatively impermeable erythrocytes (PONDER 1937, 1938) is indubitable. For cells which are permeable to ions, proteins appear to be essential.

The term protein in this connection must be understood to connote conjugated proteins, nucleoproteins, amino acids and in general any ampholyte. Proteins have usually many basic and

acidic dissociations, although the position of such ionized points is still under investigation.

The concept of the structure of protoplasm of any living cell may be illustrated by the following figures 13 and 2 and 2A (Chapter II). Figure 13 is a detailed diagram showing the side-chain or grid linkages for β -keratin, taken from the chapter by BLOCK in SCHMIDT (1938). Figure 2A shows $1/30$ of a protein molecule chain of 288 residues with a molecular weight of 38,000 (SPONSLER 1930). Figure 2 represents $1/15$ of a similar chain with about 16 residues (SPONSLER 1939). Some of these groups can be displaced readily because of loose bonds, while others are more difficult of displacement. If one keeps a diagram such as this in mind when discussing ion exchange in living protoplasm, it is easier to conceive of areas of electron dimensions perhaps, where positive or negative ions can be displaced by others coming in either through BROWNIAN movement of the molecules or by other forces specific to the atoms. It is such a concept of "mosaic" areas of negative and positive charges rather than one of "holes" in the membrane which should be pictured.

Due to the simultaneous presence of acid and basic groups of different strengths among these protoplasm molecules, there should be local fields due to both positive and negative charges, especially when ionization occurs. No one field would be permanent, and the effective range of the forces would be of atomic dimensions only, and smaller where ionization has not occurred. These electrical fields surrounding these points will attract molecules of water by their induced charge and ions of sign opposite to that of the protein directly by virtue of their charge. At the same time ions of like sign would be repelled. If the plasma membrane is relatively thin, then ions not repelled might by virtue of even a relatively low initial kinetic energy, pass through the membrane at a charged point, while repelled ions would need a larger amount of kinetic energy (e. g., heat motion) to do the same, and hence would be more or less hindered from entering or leaving the cell at this point (BROOKS, S. C. 1934). If the plasma membrane is thicker, this aspect is of less importance. However, the plasma membrane may be conceived of as a surface boundary only, with all the characteristics of surface tension and other physical properties pertinent to such structures, rather than as a discrete tangible membrane.

A protein gel is interpenetrated by a crystalloid solution, and the movement of ions in this fluid depends upon the electrical fields originating at the ionized groups of proteins.

The e. m. f.'s so produced at these regions would be local at each area, and if the areas were small, would not be individually measurable by any device now known. The experimentally measurable e. m. f.'s would have some value intermediate between the local e. m. f.'s at the different areas. This principle is shown for artificial "mosaic" membranes by HÖBER and HOFFMANN (1928). Under these conditions ions of a like sign would be exchanged through these areas. The extent of such exchanges would be closely correlated to the local e. m. f.'s which would constantly tend toward equilization of the accumulation ratios of all the diffusible ions. But because of the constant production of suitable metabolites within the cell, equilibrium would never be reached so long as the cell remained alive.

The objection made by BRIGGS (1930) that in order to keep the electrical neutrality of the protoplasm balanced, it would be necessary to suppose that there are definite periods in which the cation or anion permeability would predominate, is a permissible speculation if one considers these areas as molecular in size, or even ionic, rather than of a definite delimited microscopic or submicroscopic dimension. They would necessarily exist side by side and change only as the metabolism changes.

The protoplasm is heterogeneous and is made up of substances, (mainly nitrogenous) having different isoelectric points, each with one or more dissociating groups, each group with its own dissociation constant. There are probably present regions, or lines of travel, some cation-permeable and some anion-permeable. The predominance of one or the other type will depend upon the nature of the cell and upon the metabolic processes. The mosaic structure of protoplasm visualized by HÖBER and HÖBER (1928) no doubt is of this general description. PUMPHREY (1932) visualizes membranes as containing pores with adsorbed ions on their walls, principally anions, and the lumen of the pores, if sufficiently small, as containing principally cations which can be displaced, thereby accounting for the relatively high mobility of cations in the membrane. This appears to be a rather naive mechanical concept for which we submit Figures 13, 2 and 2A of protein or other molecules. Pores must be replaced by inter- or intramolecular spaces.

The degree of dissociation will be the limiting factor as to whether or not the particular region will be cation- or anion-permeable with reference to its surrounding regions. Such ions so produced within the cell will be available for exchange with ions in the external solution, as for example, with K^+ ion.

The K ion will enter the protoplasm, in the case of these plants, before the sap, and the ions available in the protoplasm will be exchanged before those in the sap. Such a gradient in-

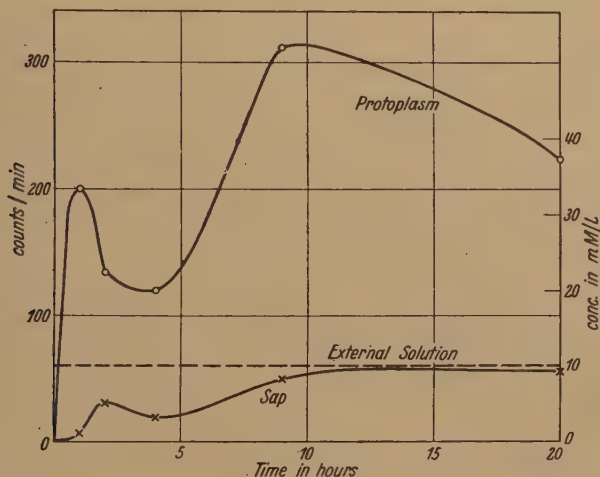


Figure 14. Rate of penetration of K^*Cl into sap and protoplasm of *Nitella*.

wards from protoplasm to sap has been shown experimentally (S. C. BROOKS 1937, 1938). A relatively small exchange of ions occurs between protoplasm and sap, but most of the exchange will at first occur between protoplasm and the exterior, since the concentration of basic ions in the protoplasm is high. No measure of this concentration has as yet been directly made. Time curves taken from the work of S. C. BROOKS on the penetration of radioactive substances into *Nitella* show that accumulation takes place in the protoplasm very soon after the plants are placed in solutions and that accumulation is found to a marked degree in the protoplasm even before it can be demonstrated in the sap (See Figure 14).

It is probable that exchanges occur with ions attached to the proteins until the concentrations on both sides are equalized.

The picture is perhaps best illustrated by a continuous oscillation and exchange of ions until a statistical equilibrium has been attained¹).

Experiments with radioactive elements. A more complete picture of the experiments illustrating this ion exchange hypothesis (S. C. BROOKS 1937, 1938a, b, c) follows:

Cells of *Nitella clavata* were placed in solutions of radioactive isotopes of the alkali metals for periods up to 24 hours. The movement of ions was followed by GEIGER-MÜLLER (β -particle counter) counts in preparations containing corresponding radioactive isotopes. (See also page 179.) For example, KCl contains about 93.3% ^{39}K , 6.7% ^{41}K and a trace of radioactive ^{42}K produced by neutron bombardment. Portions of 200 ml. of 0.01 M K^+Cl were usually prepared with activities of about 20 μC . Other isotopes show different proportions of β and γ activities and appropriate modifications were used. Na, Rb and Br besides K were studied (Figure 15). For details of technique see BROOKS (1938a). Control of light has been introduced in later experiments. The curves show three phases in the absorption processes, an immediate and sharp increase (Phase I), a sharp decrease (Phase II), and a gradual increase leading to equilibrium with final death in which a decrease occurs (Phase III).

Phase I, characterized as ion exchange, hereafter is considered to be "induced accumulation" following STEWARD's (1937) terminology, and is distinguished from Phase III, which is "primary accumulation". Phase I consists of replacement of removable similar inorganic ions in the protoplasm as shown by the appearance of isotopes therein within 1—5 minutes in concentrations exceeding those of the corresponding experimental solutions. Apparently this intake continues, possibly complicated

¹) It has been suggested that the electrical conductivity of solutions of strong electrolytes is due to the discontinuous movement of ions from one to another of several relatively stable positions. The movement of ions (with or without electrical fields) through the plasma is conceived of the same type — a discontinuous movement of ions. Two adjacent ions probably exchange positions during their heat movement with simultaneous ionization. The net effect of such movements would dissipate their concentration gradients. Such movements probably occur at high frequency, at least several million per second.

by Phase III, for several minutes or up to 2 or 3 hours. This exchange depends on the prior presence of measurable concentrations of such ions in the protoplasm at the start of the experiment. Cells removed from the normal environment, pool water, show much less such intake than similar cells allowed to take up ions from dilute solutions (e. g., KCl, of 0.01 M for 24 hours). For example, the protoplasm, thus enriched, showed in 5 minutes

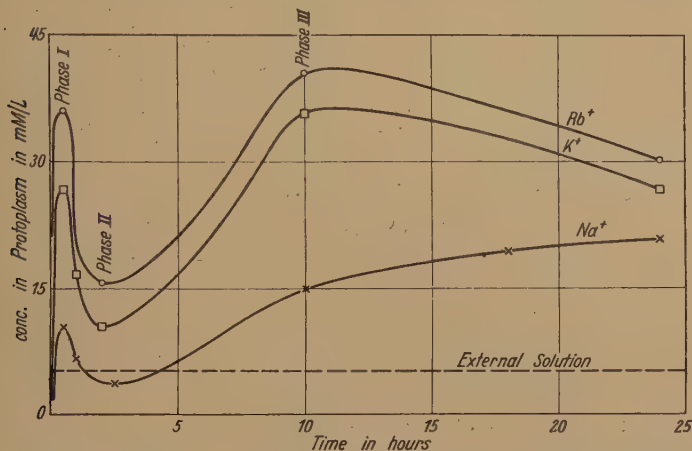


Figure 15. Rate of penetration of Na⁺, Rb⁺ and K⁺ into *Nitella*.

a concentration of (0.035 M from a 0.01 M K⁺Cl solution, while control cell-protoplasm in a solution of 0.01 M) of inactivated KCl showed no penetration.

Furthermore, it appears that this replacement approximates expected amounts when experimental solutions of different concentrations are used. Replacing K from KR by K⁺, forming K⁺R, where R is an unknown fixed anion of the protoplasm and K⁺ comes from an excess of K⁺Cl immersion solution, we may expect the activity of the protoplasm to be parallel to the external K⁺Cl concentration. If the active mass (The term "active mass" denotes the total number of reacting (univalent) groups of the protoplasm) and the immersion solution were equal, the amount of K⁺ bound by R would vary as the square root of the concentrations of the immersion solution. The experimental conditions lie between these extremes, and the concentrations found in the protoplasm lie between the limits thus indicated. Thus, when the

immersion fluids were 0.005 and 0.02 M K^+Cl^- , the first maximum concentrations of K^+ in the protoplasm (using means of 5 experiments) were 0.051 M and 0.090 M. Similar results were given with other ions.

The induced accumulation as here observed means that at least some of the alkali metal ions are free to enter and leave the protoplasm, reaching (mobile) equilibrium for this process in a time of the order of 5 minutes to 2 hours. The protoplasm is therefore highly permeable to such ions, contrary to our traditional ideas. The slow entrance of ions observed with osmotic methods is illusory and in this case depends upon limiting factors other than the permeability of the protoplasm or its surface layers (plasma membrane).

Common experiments (of which the authors are also guilty) on protracted studies in which the concentrations of these ions in the sap of such cells were determined, are also illusory. The limiting factor in both primary and induced intake of ions is the provision of ions within the protoplasm (not the sap) for counter movement. In both cases the observed intake is originally due to organic ions which result from metabolism. The relation between metabolism and ion intake has been recognized by OSTERHOUT (1926), BROOKS (1929), STEWARD (1933), LUNDEGÅRDH and BURSTRÖM (1933) and others. But neither the precise nature of the metabolism nor the identity of the ions are as yet known.

Location of radioactive ions absorbed. In the experiments of S. C. BROOKS (1939) an experimentally measured amount of ions from the external solution had entered the protoplasm by induced accumulation. Calculations were made to determine whether or not the ions so exchanged may have been present in an aqueous matrix of the protoplasm, or may have combined with the components of the protoplasm as a whole, or both. In these calculations, BROOKS has taken as a basis for measurement a series of internodal cells of *Nitella* which yield 0.02 cm. of sap. Details of these calculations are here omitted. However the following assumptions are made, overcompensating for the maximum dimensions as a margin of safety; that the plasma membrane is a monolayer of proteins 40 Å thick; that the combining capacity of these proteins equals that of such a protein as serum albumen; and that at pH 7.5, a single SVEDBERG molecular

weight unit (M. W., 35,000) of this protein can combine with 13 alkali metal ions; that the volume of a SVEDBERG unit can be taken as 3.35×10^{-20} cm.³ Under these conditions, the surface of these units would furnish salt linkages for 5.22×10^{-10} equivalents at pH 7.5. At pH 14 as a maximum, the equivalents would be 12×10^{-10} . A layer of such a fatty acid as stearic acid, 40 Å thick, would afford linkages at most for 24×10^{-10} equivalents. The thicker plasma membranes or nerve sheaths, 170—180 Å in diameter, described by SCHMITT (1938, 1939) are believed to consist primarily of bimolecular layers of fatty acids mixed with smaller amounts of lipins, sterols, cerebrosides and interlarded with protein monolayers. If the plasma membrane corresponded to this dimension, proteins at pH 7.5 would accommodate 23×10^{-10} equivalents, and fats would accommodate 114×10^{-10} equivalents. In contrast to this, BROOKS found that *Nitella* protoplasm contains about $2-8 \times 10^{-7}$ equivalents, i. e., about 20—1600 times as much as could be accommodated as counter ions in the surface layer of the protoplasm. See BROOKS, S. C. (1939). Therefore the first reaction of the salt with the cell concerns the whole cell rather than any superficial process, i. e. in the plasma membrane. We may therefore make calculations neglecting that minute amount of the ions bound by the plasma membrane.

The volume of whole protoplasm of the standard amount of *Nitella* would be 0.01 cc. and this probably contains about 70—90 per cent of water and crystalloids, the remainder containing proteins and fats and other water-insoluble materials. Provisionally we may assign 6—20 per cent of the 0.01 cc. of protoplasm, viz., $0.6-2 \times 10^{-3}$ cc. to proteins, and may calculate that about $2-6 \times 10^{16}$ SVEDBERG units are contained in this protoplasm. If all the salt linkage available at pH 7.5 were occupied by alkali metal ions, this ion content of the protoplasm would be 6.6×10^{-6} equivalents, while it would be at pH 14, 28×10^{-6} equivalents.

But since $2-8 \times 10^{-7}$ equivalents enter the protoplasm, this approximate correspondence acquires significance. The ten-fold discrepancy which exists might be supposed to be due to the fact that a large part of the protoplasm consists of chloroplasts which afford relatively little salt-combining capacity. Their volume appears to amount to 50—90% of the whole protoplasm

of *Nitella*¹). However some chloroplasts contain significant proportions of proteins (GRANICK 1938), so that their relative salt combining powers are still unknown. One may also consider the possibility that a portion of the protoplasm may not enter into cation exchange, a differentiation possibly related to the moving and stationary layers of the protoplasm. Finally it may be that only a part of the potentially available salt linkages actually enter into cation exchange. We may account for a large portion of these ions found in the protoplasm as being combined with the protoplasmic constituents, that is, retained close to the oppositely charged group, though remaining dissociated. The remainder is free in the protoplasmic matrix as one of a pair of inorganic salt ions. This free portion may include ions accumulated because of primary accumulation, utilizing the exchange of organic ions produced by the cellular metabolism for the inorganic ions taken in. Although it seems premature under the present conditions to allocate these ions more than tentatively between these classifications, still as a first step, we can assume that primary accumulation is generally practically absent during the first period of 15—60 minutes, and accordingly consider that the total content of the protoplasm, minus that portion commensurate with the immersion fluid, would be combined with the protoplasmic constituents. Proceeding in this way we may estimate that the amount of ions combined with the protoplasmic constituents varies from none for bromide through many values around 10—30 up to 65 mEq. liter⁻¹ as a maximum.

The ion exchange nature of this intake and binding of ions is quite apparent. In combining with these proteins, etc., these radioactive ions must replace other ions; the (inactive) ions present at the beginning of the experiment would include the hydrogen ion and inorganic ions such as Na, K, and, in the case of Br*, as Cl. In the same way we may guess that the removability of these ions is a factor in the exchange for the applied radioactive ions. Table XXIX supports this formulation. The ionic exchange basis of this induced accumulation has been recognized by many authors, including BÖNNIGER (1909), BONNET (1922), HEVESY (1923), STILES (1924), BROOKS (1929), GENAUD (1930), GENEVOIS (1930), STEWARD (1935), and LUNDEGÅRDH (1938).

¹) Original observations.

Table XXIX. Summary of concentrations of radioactive salt being absorbed within 5, 15, or 30 minutes under specified conditions in the protoplasm and in the sap. Only Exp. K and Rb 12 received continuous illumination, and all potassium experiments and Rb 10 and 11 had a controlled temperature of 15° C. (Taken from Brooks, S. C., 1939)

Experiment		Pretreatment (Inactive ions)			Immersion fluid (Radioactive ion)			Obs'd after mins	Conc. in	
Radioactive ion and exp. no.	No. of trials	Salt used	Conc. used mEq/l	Duration hrs	pH	Salt conc. mEq/l	CaCl ₂ conc. mEq/l		Proto plasm mEq/l	Sap mEq/l
Na 5	4	KCl	10	15	—	20	—	15	45	5
K 9	2	KCl	10	17½	6.18	20	0	5	17.5	6.0 ¹
	1	„	10	17½	6.18	10	0	5	11.0	2.0 ¹
	2	„	10	17½	6.18	5	0	5	9.0	0.5 ¹
K 10	1	KCl	10	16	5.97	20	0.4	15	85	24.0
	1	„	10	16	—	10	0.4	15	14	3.0
	1	„	10	16	6.37	5	0.4	15	33	5.0
	1	None	10	16	—	5	0.4	15	35	6.0
K 11	1	KCl	10	15	5.6	20	0.4	5	59	0.5
	1	„	10	15	6.0	10	0.2	5	35	1.0
	1	„	10	15	5.8	5	0.1	5	12	2.5
	1	None	10	15	6.0	5	0.1	5	—1.0	0.0
K (and Rb) 12	2	None	10	0	7.35	5	0.1	15	22.5	0.0
Rb 5, 6 and 7	3	NaCl	10	36	7.5	30	—	30	28	8
	3	„	10	36	to	10	—	30	19	6
	3	„	10	36	8.1	3.4	—	30	9	3
	3	None	0	—	—	10	—	30	20	7
Rb 2, 3, 4 and 9	3	KCl	10	10	7.8	30	—	30	74	6
	7	„	10	to	to	10	—	30	36	10
	3	„	10	27	8.2	3.4	—	30	27	4
	3	None	0	—	—	10	—	30	22	3
Rb 8	1	RbCl	10	12	7.0	30	—	30	56	48
	1	„	10	12	to	10	—	30	57	50
	1	„	10	12	7.5	3.4	—	30	11	15
	1	None	0	—	—	10	—	30	11	10
Rb 10	5	None	0	—	7.3	5	0.1	30	55	5
(K and) Rb 12	2	None	0	—	7.35	5	0.1	15	17	0
Br 3	2	None	0	—	5.8	10	0.2	30	sl. < 1	0
	2	„	0	—	5.45	10	0.0	30	sl. > 1	0

¹) The sap concentrations thus designated referred to 30-minute readings.

Table XXIX shows that the rate of penetration of these ions in induced accumulation lies between practically zero for bromide and 1.76×10^{-7} GM. cm. $^{-2}\text{hr}^{-1}$ for potassium with 20 mEq. liter $^{-1}$ of K*Cl in the immersion fluid. (Exp. K 11). Most of the values lie around 1×10^{-7} GM. cm. $^{-2}\text{hr}^{-1}$. These intrinsic rates of penetration exceed materially the previously reported values (COOPER, DORCAS and OSTERHOUT 1929, BROOKS 1932). The value given for the rate of entrance of rubidium into *Valonia* (BROOKS 1932) approaches these values, but the conditions are so different as to make comparisons impossible. The present difficulty encountered in evaluating the motive forces limits the values of these figures, but we are forced to recognize that ions penetrate the protoplasm quite rapidly, like most non-electrolytes and water, for which the rates of penetration are of the order of 10^{-7} GM. cm. $^{-2}\text{hr}^{-1}$.

The fact that Br* failed to enter the protoplasm during the induced accumulation phase might be interpreted as due to the absence of material numbers of basic linkage. This condition might well be correlated with the fact that practically none of the basic groups are dissociated at the prevailing pH's of the protoplasm, viz., ca. pH 7.0. We prefer however to refrain from committing ourselves to this apparently attractive hypothesis.

Experiments reported by MULLINS and BROOKS (1939) show the occurrence of exchange through the surface of the cell of like-charged ions, (so-called "selective ion exchange") so that this replacement principle applies to this phase of ionic movement.

Loss of ions from the protoplasm or loss phase. The second phase of intake (Phase II) is one in which ions are lost from the protoplasm. It seems to be suggested by the subsequent course of the absorption curve that this loss of radioactive ions is the same as the recurrent losses during primary accumulation which is discussed below, namely replacement of absorbed and combined radioactive ions by organic ions produced by the metabolism of the protoplasm. The experiments of MULLINS (1939) show that with radioactive Na having different activities and identical molalities, there occurs less penetration in concentrations of Na* with higher activities. A more probable explanation can be suggested in connection with the Phase I or induced ion exchange. At the time of immersion of cells in a solution of radioactive salt having a relatively high concentration (e. g.

0.01 M) a store of removable organic ions within the protoplasm was present, and the introduction of inorganic ions in the immersion would provoke an ion exchange, thus removing the organic bases from the cell. The well-known stimulation of metabolism by K, or the removal of end products of metabolic reactions would enhance metabolism. If this sequence requires considerable time to produce these waste products, then this production would displace a certain fraction of the previously accumulated radioactive ion. Phase II, with loss of radioactive ion, occurs more quickly when K or Rb are used, as compared with experiments with Na.

The ion exchange mechanism of "induced" intake of ions is supported by experiments on exosmosis of ions (MULLINS, unpubl.). Radioactive ions were accumulated by *Nitella* cells and the radioactivity of intact single cells was determined. These cells were transferred to distilled water or 0.01 M alkali metal salts (not radioactive). Little or no loss of radioactivity of cells occurred when they were in distilled water, whereas a decrease in radioactivity of cells occurred when they were lying in salt solutions. This change occurred within a few minutes, and at rates varying according to the particular accumulated ions, and the salt used as an immersion fluid during this exosmosis of the accumulated ion.

The reversibility of this ion exchange and the indispensability of counter ions is well shown here. The same exchange had been shown by MAZIA (1938). The Ca ion will not come out of the protoplasm of *Elodea* during 14 days immersion in distilled water, whereas the addition of Na or K salts (either oxalates or chlorides) will cause its release from the cell. Some permeability constants are given in Table XXX.

Table XXX. Some Permeability Constants
for Radioactive Ions

Ion	P (M/cm ² /sec) $\times 10^{-8}$
³⁷ Rb ⁸⁶	8.50
¹⁹ K ⁴²	2.63
¹⁴ Na ²⁴	.48
³⁵ Br ^{80, 82}	.72

After S. C. BROOKS, unpublished.

Further confirmation of the ion exchange theory has been shown by the experiments with radioactive K of JENNY and OVERSTREET (1938) in which they noted ion exchange between roots and clay particles. No K^* was lost when the roots were placed in distilled water, but when alkali chlorides were used in the solutions, rapid removal of K^* from the tissues took place. Ion exchange between clay and root was much greater than between salt solution and root, a difference which the authors believe to be due to adsorption layers adjacent to the roots and soil particles. Preliminary results of JENNY and OVERSTREET show that certain clay colloids are precipitated by radioactive K while ordinary K has no such effect. This suggests that there may be an effect upon the charge of these clays which induces precipitation. Further experiments should clear up this point. See also Manual of Radioactivity by HEVESY and PANETH (1938).

The rapid intake of radioactive K ions has also been observed by GREENBERG, JOSEPH, COHN and TUFTS (1938). They fed it to white rats and noted its absorption from the gastro-intestinal tract. In the case of radioactive phosphorus (COHN and GREENBERG 1938) the rapid uptake within the first few hours by the various tissues of the rat was again seen. In these experiments whole organs are used for analysis and no effort is made to locate the specific path of the intake of the radioactive ion in the specific region of the cell. Here no central vacuole occurs, but inter-cellular spaces do enter the picture. Accumulation of these ions is selective for certain tissues. SCOTT and COOK (1937), using Na^* , showed that the hematopoietic system, lymphoid tissue and intestinal epithelium are especially active in this respect. HAMILTON and ALLES (1939) confirmed this for man. See also HAMILTON (1937).

The absorption of phosphorus was first noted by CHIEVESY and VON HEVESY (1935). The localization of phosphorus in bones has been shown by DOLS and JANSEN (1937), COOK, SCOTT and ABELSON (1937), PERLMAN, RUBEN and CHAIKOFF (1937), COHN and GREENBERG (1938).

The rapid absorption of radioactive iron from the upper intestinal tract was shown in the case of anemic dogs, as opposed to non-anemic dogs, and the rapid appearance of radioactive iron in the plasma and red blood cells (HAHN, BALE, LAWRENCE and WHIPPLE 1938).

All these experiments deal with whole tissues or organs rather than with the location of the radioactive element in the ultimate region of the cell as was shown for *Nitella*.

JACQUES and OSTERHOUT (1934) and JACQUES (1936) state that *Valonia* cannot endure an external pH as low as 5.5, whereas if *Nitella* is placed in this pH, the rate of increase of K^+ is practically independent over the range from 5.7 to 8.5.

HOAGLAND (1934) has shown that *Nitella* and roots of barley plant can accumulate both K and Br when the pH of the external solution is more acid than that of the vacuole. These experiments have been used to discount the existence of pH gradients as the interpretation for accumulation of ions. (See also ROBBINS (1939) on excised roots.) However, in the light of the more recent experiments on protoplasm, we believe that it is evident that the pH of the sap is not the important factor, and that the concentration of H ions (rather than pH) and/or others available for exchange must be greater in the protoplasm than in the external solution if penetration goes in the direction of outside to inside. The pH of the protoplasm is presumably about 7.0 whereas that of the sap is 6.0. Since each kind of living cell has its own special chemical composition, which differentiates it from other cells, these will be instrumental in affecting the ion exchange between the outside and the inside of the cell. Until we do know more concerning these unknowns, we cannot answer the question as to why the different cells have different rates of intake of ions.

A study of the isoelectric points of some of the constituents of protoplasm itself, and a better knowledge of what kind of proteins, amino acids and so forth make up the various kinds of protoplasts of cells may help to answer these questions.

The third phase or that of primary accumulation can be explained on the basis of exchange of organic cations (or anions) for the radioactive ions provided in the immersion fluid. It seems probable that these organic ions are produced in the metabolism. This general concept has been advanced by many workers (NATHANSON 1904, HOAGLAND, HIBBARD and DAVIS 1926, BROOKS 1929, STEWARD 1933, LUNDESGÅRDH and BURSTRÖM 1933). These products may include many substances generally dismissed as "wastes", but their fundamental, or even crucial, importance is apparent in connection with the ion exchanges generally occurring.

It may be pointed out that the mutual exosmosis of "waste" products, possibly toxic, and endosmosis of salt ions reveals a biologically valuable process. Its protective function has been generally neglected and merits investigation.

The experiments shown in Figure 16 suggests the partial or complete abolition of those metabolic reactions implied by ion intake. It is quite possible that, while ether killing completely

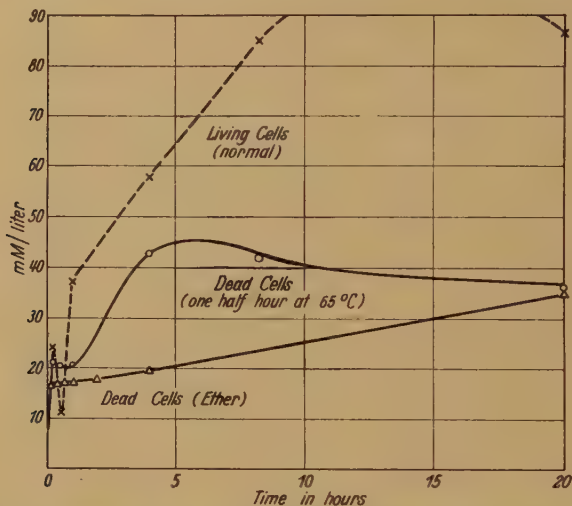


Figure 16. Penetration of 0.01 M Rb^*Cl into protoplasm of *Nitella*, showing differences between living cells and cells killed by heat and by ether.

Abscissae, time in hours; ordinates, millimols of Rb^*Cl per liter.

inactivates the enzymes responsible, heating at 65° for 0.5 hour may leave at least part of the original potency of these enzymes.

The periodical nature of this intake may depend upon inter-related effects of the stimulating influence of the ions and the relatively slow diffusion out of the organic ions in exchange for the inorganic ions. Differences in the velocity of these processes may lead to periodic processes. In the course of the consequent phenomena there recur periods in which the already absorbed and combined radioactive ions are lost to the cell. This loss culminates in the previously discussed "first minimum". Alternate with these loss phases occur periods of primary accumulation.

The first loss phase was observed by PANTANELLI (1918) using *Lupinus albus* and *Vicia faba* roots, *Lupinus* and *Allium cepa* plants, *Valonia utricularis* and *Saccharomyces* sp. in experiments with NH_4^+ , Na^+ , Mg^{++} , Ca^{++} , Ba^{++} , Zn^{++} and Cl^- , Br^- , NO_3^- , SO_4^{--} , and H_2PO_4^- in various combinations. The failure to detect later loss phases may be due to their absence in these experiments, or possibly because of the diminishing frequency of observations. However, a final decrease was noted often. This work confirms our conclusion that all these features are essentially free from the radioactivity of the ions in our work.

Recent work of COHN and COHN (1939) on the entrance of Na^* into red blood corpuscles of the dog appears to conflict with the present work. The concentrations of Na^* in these cells show only a smooth approach to a simple equilibrium, with no induced accumulation, no loss phase and no periodic primary accumulation. Since the first readings were taken after 8 minutes in contact with radioactive Na, it appears that the first initial phase of induced accumulation which occurs during the first two minutes in the case of plant cells and yeast (MAZIA, in unpublished experiments) may have been overlooked. Although many aspects are still partly understood, it should be kept in mind that (a) the erythrocytes have reduced metabolism; (b) the dog erythrocytes admit sodium principally, as compared with, for example, the human erythrocytes which admit principally potassium; (c) the principal organic constituent of erythrocytes is hemoglobin, a protein whose isoelectric point is about 6.7 as compared with those of most proteins, $4.8 \pm$. This protein would combine with less base than most proteins would at such a pH as 7.0—7.3, the pH of most cells; and that (d) other animal cells, such as *Urechis* eggs and sperm (unpublished results) do show these features which are lacking for dog erythrocytes.

Rôle of sap. Recognizing the primary rôle of the protoplasm in absorption and accumulation of ions, we are interested also in the wholly passive rôle of the sap. During the first 1—3 hours, the sap normally contains no or very small amounts of newly absorbed radioactive ions. With the further duration of the experiment, ions appear in considerable amounts. When, e. g., after 12 hours, primary accumulation has occurred in the protoplasm, the sap, either with or without any special ion permeability, obtains ions in concentrations notably exceeding those

present in the immersion fluid. A lag in the increase and decrease of ions in the sap as compared with similar changes in the protoplasm is often noticeable. This seems, therefore, to show that similar hypertonic solutions were present in the protoplasmic matrix in advance of changes in the sap. This situation has already been made probable in the previous sections of this paper. However, no further attempt is made here to demonstrate this.

The amount of radioactive ion in the sap has significance in interpreting the results obtained with intact cells. The standard volume of sap corresponding with the standard amount of the protoplasm (0.01 cc.) is 0.02 cc. Assuming similar volume relations in the experiments on intact cells, we must subtract an appropriate amount from the gross amounts so observed. Although the subtractions have a considerable effect, still the major part of this amount is demonstrated to be due to accumulation in the protoplasm. It is not practicable at the present time to suggest metabolic mechanisms or the nature of the organic ions in question, a subject at this time under investigation.

Previous work on sap of coenocytes like *Valonia*, *Nitella*, and *Halicystis* do not afford measures of the state of metabolism and of permeability to ions of these living cells. The protoplasm itself is the important feature.

The advantage of using a plant such as *Nitella* or *Valonia* is not so much to compare concentrations in the sap with those in the external solution, as has been so frequently stated, but to be able to see what substances penetrate through the protoplasm into the sap because the sap and the protoplasm can be separately analyzed. This cannot be done in microscopic cells to such an accurate quantitative degree.

A summary of some permeability constants for ions and accumulation ratios for various ions have been calculated and collected in Tables XXXI and XXXII.

Bioelectric phenomena. Potential differences are responsible for electrophoresis, or migration of ions. It is believed that p. d.'s exist primarily across such organized layers as the plasma membrane, but if p. d.'s occur elsewhere, they must still cause electrophoresis.

The possible sources, considered from the physico-chemical viewpoint, of the potential differences (p. d.'s) encountered in biological material would include the following: —

1. P. d. due to the motion or mobility of ions, a) diffusion p. d.'s as given by equations of NERNST (1888) and HENDERSON (1907). b) Membrane p. d. as given by DONNAN (1911). c) Phase boundary p. d.'s due to the assumed asymmetrical distribution of ions, due to differences in partition coefficients of ions. DONNAN equations would be applicable to this source. See also NERNST (1892).

2. P. d. due to the motion of electrons, if one may assume the metallic behavior of any constituents of the protoplasm ("first class conductor"). Such behavior can be postulated for other than metals, e. g., by LUND.

It seems apparent that any observed p. d. in tissues and cells may be attributable to more than one of the above sources.

Furthermore it must be emphasized that no distinction can be made between the usually considered inorganic ions, and the organic ions almost surely provided by the metabolism of the cells or tissues.

The electrokinetic potentials ("ζ p. d.") cannot appear as p. d. as such, but only by virtue of an impressed electromotive force which is due to the above sources, which brings about a mechanical result, viz., motion of a particle or flow of a fluid, d) forced orientation of ions as in monolayers. RIDEAL (1933), YAMINS and ZISMAN (1933).

Although no direct measurements of potential differences have been made across the plasma membrane alone, nevertheless we can deduce that such exist from experiments closely associated with the plasma membrane. As soon as an electrode (i. e., salt bridge) is placed within the protoplasm of a cell, the protoplasm immediately form a membrane or "plug" around it, so that one cannot actually measure p. d.'s in the plasma membrane as such. However, the fact that there are potential differences between the protoplasm under these conditions and the outside solution, is proof that such differences must exist either across the plasma membrane or across the plug, or both. See TAYLOR and WHITAKER (1926), OSTERHOUT, DAMON and JACQUES (1927), JOST (1927), GICKLHORN & UMRATH (1928), BROOKS and GELFAN (1928), UMRATH (1930, 1938), and KOPAC (1935). Moreover, such a well-established phenomenon as the DONNAN Equilibrium points to the conclusion that potential differences on the two sides of the plasma membrane occur.

Table XXXI. Some Permeability constants for ions

In this table the ion in bold face type is the one whose permeability is being given. Almost all the values have been recalculated, whenever possible, to have the values in comparable units. The pH values selected are those most closely resembling the pH of the normal external environment of the organism. Illumination is always in diffuse daylight of the laboratory. All calculations are based on the time required for an ion to appear in the sap. The rate of penetration appears somewhat independent of external concentration and hence data are not calculated on the basis of per unit external concentration.

Organism	Tissue or Cells	t °C	External Solution	pH	P M/cm ² /sec	Observer
<i>Valonia ventricosa</i> J. G. AGHARD	whole cell	22—28	RbCl .006—.025 M	—	1.86×10^{-10}	BROOKS (1932)
<i>Valonia macrophyssa</i> KÜTZ .	whole cell	—	KCl 0.024 M NaCl 0.024 M	— —	7.3×10^{-11} 1.53×10^{-11}	COOPER, DORCAS and OSTERHOUT (1929)
<i>Nitella clavata</i>	whole cell	21—22	K⁴¹Cl 0.01 M	5.6	2.2×10^{-12}	BROOKS (1938)
<i>Nitella flexilis</i>	whole cell	19.5—21	KCl 0.01 M	7.0	1.7×10^{-12}	JACQUES and OSTERHOUT (1935)
<i>Nitella</i>	whole cell	20—22	KBr 0.06 M KCl 0.05 M	5.0—5.4	3.0×10^{-14} 1.2×10^{-12}	HOAGLAND and DAVIS (1929, 1923)
<i>Valonia macrophyssa</i> KÜTZ	whole cell	—	NaI 0.04 M	8.2	3.0×10^{-13}	JACQUES (1937)

Table XXXII. Accumulation ratios for various ions¹⁾

Organism	Ion	External Con- centration M/l	pH	Ratio ¹⁾	Observer	
<i>Nitella clavata</i> (in normal pond water)	Na ⁺	.01	8.8	46.	HOAGLAND and DAVIS (1923)	
	Mg ⁺⁺	.0034	8.2	10.		
	Ca ⁺⁺	.0016	8.8	13.		
	Cl ⁻	.0009	8.8	100.		
	PO ₄ [≡]	.000004	8.8	870.		
	SO ₄ [≡]	.0007	8.8	26.		
<i>Valonia macrophysa</i> (in normal sea water)	K ⁺	.010	8.5	51.	STEWART (1937)	
	Na ⁺	.481	8.5	.235		
	Cl ⁻	.555	8.5	1.14		
<i>Valonia macrophysa</i>	I ⁻	.04	8.5	.037	JACQUES (1938)	
<i>Nitella clavata</i>	Br ⁻	.06	5.0—5.4	.74	HOAGLAND and DAVIS (1929)	
<i>Halocystis ovalis</i> (Lyng.) (in normal sea water)	Na ⁺	.480	8.5	1.08	BROOKS (1932)	
	K ⁺	.011	8.5	1.2		
<i>Valonia ventricosa</i> <i>Nitella clavata</i>	³⁷ Rb ⁺ ⁸⁵	.006	8.4	Sap Ratio 39.	Protoplasm Ratio ¹⁾ > 500.	BROOKS (1935)
	¹⁹ K ⁺ ⁴²	.01	8.0	10.5	30.	BROOKS (1938a)
<i>Nitella clavata</i>	³⁷ Rb ⁺ (?)	.015	8.8	2.0	8.3	BROOKS (1938b)
			6.6	2.5	10.5	
	³⁵ Br ⁻ ⁸²	.01	8.8	.8	.9	
			6.6	1.5	11.	
	¹¹ Na ⁺ ²⁴	.01	8.8	.1	1.5	
			6.6	.2	2.0	

¹⁾ The ratio is defined as the concentration of ion in the sap over the concentration of ion in the external solution. Maximum observed values are used.

²⁾ The concentration of ion in the protoplasm over the concentration of ion in the external solution.

The driving force for the movement of ions may be designated by the following equation:

$$F = \frac{d a}{d x} + \frac{d \pi}{d x}$$

where a = the activity in moles, π = the potential in volts, and x = thickness in cm. "P. d." refers to a difference in potential energy of a unit plus or minus charge between two points. Work is done or yielded if the unit charge is moved between two points, i. e., the charge has potential energy relative to a selected position. In the case of cells, metabolism is requisite for the maintenance of $\frac{d a}{d x}$.

The correlation between metabolism and p. d.'s and accumulation have been studied in roots of *Allium cepa* by LUND and ROSENE (1935) and ROSENE (1934) and other publications by the same authors; by PREVOT and STEWARD (1936) in noting the reduction of methylene blue in elongating region of active root hairs, and the absorption of salts in primary roots as compared with older roots of barley. (See CRAFTS and BROYER 1938).

Biological potentials occur through p. d.'s. The mechanism of p. d. production is due to the difference in movement of two ions of a salt, according to the well-known diffusion potential equation

$$E = \frac{RT}{F} \cdot \frac{u - v}{u + v} \ln \frac{c_1}{c_2} \quad (\text{for univalent salts}) \quad (I)$$

in which E is the electromotive force, u and v the transport numbers for cations and anions and c_1 and c_2 the two concentrations of a solution, R = gas constant, T = absolute temperature and F the Faraday.

In artificial membranes (such as layers of gels or similar structure) the equation must be modified as shown by MEYER (1936) and TEORELL (1936). The values of u and v in Equation I refer to free diffusion of ions in a homogeneous solvent. Heterogeneous systems occur in membranes and the concentrations of ions are affected by partition between two or more phases and by membrane equilibria (DONNAN 1911). By introducing appropriate corrections, one may calculate the p. d.'s under different conditions of absolute and relative concentrations of ions on the opposite

sides of the membrane, the pH, and the membrane material. Using the notation of MEYER the equation is

$$E = \frac{RT}{F} \left[u \ln \frac{x_2 + A_u}{x_1 + A_u} + \frac{1}{2} \ln \frac{(x_1 + A)(x_2 - A)}{(x_1 - A)(x_1 + A)} \right] \quad (II)$$

where $u = \frac{U_k - U_A}{U_k + U_A}$, and $x = \sqrt{4c^2 + A^2}$, in which $A = \text{con-}$

centration of immobile ions in the membrane, (selectivity constant), and $c = \text{concentration of the salt bathing the two sides (denoted by subscripts 1 and 2) of the membrane. This equation can be calculated from the HENDERSON p. d. plus the DONNAN p. d.:$

$$E = E_{\text{Hend}} + E_{\text{Don1}} - E_{\text{Don2}}. \quad (III)$$

The calculated p. d.'s yield curves like the observed curves and in this way differ from the classical diffusion potential predictions. Moreover, this treatment accounts for the so-called electrode behavior of living membranes, thus reconciling the two interpretations.

Artificial membrane potentials have been studied by MICHAELIS (1925), TEORELL (1937), K. H. MEYER (1937), NORTHROP (1929), LOEB (1919, 1922, 1922a). A large amount of work has been done by BLINKS and OSTERHOUT and colleagues, DAMON and OSTERHOUT (1930) et seq., DAMON (1932) and up to the present time, (See J. Gen. Phys. from 1929 to the present date) on polarization capacity and resistance of *Valonia* and allied cells and the effects of current flow on bioelectric potentials. BLINKS concludes that the "cells are permeable to non-ionized lipid-soluble materials but still display electrical effects ascribable to ionic mobility".

Single regions of plant cells have been studied by GICKLHORN and UMRATH (1928) in which microelectrodes were inserted and potential differences observed. See also BAILEY and ZIRKLE (1931) on the differentiation of regions by vital staining, indicating differences in potential at isolated portions of the cell.

UMRATH (1938) using smaller electrodes than BLINKS and inserting them into the protoplasm of *Valonia macrophysa* found that the protoplasm has a negative potential towards sea water on the one hand, and towards sap on the other; that it is more negative towards sap than towards sea water. This seems to be additional evidence for the hypothesis of ionic exchange in that

a high gradient is established in the protoplasm from which ions descend into the sap.

LUNDEGÅRDH (1938) concludes that the removal of H^+ ions by K^+ ions is an adsorption process. He believes that he is measuring the surface effect. The lower end of a root was bathed in different salt solutions (KCl , Na , Li , Ca , etc.) at different pH's from 3.0 to 8.0. The characteristic p. d. was assumed within 1—2 minutes. It appears that this p. d. is a manifestation of ion exchange (Phase I). Connection was made to non-polarizable electrodes with KCl -agar and solutions. A salt solution of a specific pH made contact with the cut end of the root, and under these conditions this surface acted as an indifferent surface.

Oxidation is probably at the basis of the p. d.'s produced through the ions of strong and weak acids and bases. Ions are accumulated probably because of potential differences. But both cations and anions are accumulated, and since no one p. d. is able to accomplish both of these processes, therefore there must be local differences in potential which may be opposite at different points. In the case of *Nitella* there are large differences in potential up to 30 mv. observed between two spots (See OSTERHOUT and HILL 1935). This observation agrees with the concept of HÖBER and HOFFMANN (1928) that there exists a mosaic of cation- or anion-permeable areas in the membrane.

LUND and his school have suggested the possibility that oxidation-reduction reactions are responsible for the observed p. d. In this work he has emphasized certain sources of error often forgotten, namely, a) the use of injured or stimulated material, and b) the failure to use symmetrical electrodes. By this LUND implies any suitable electrode making connection by a "salt bridge", which is identical at both points of contact with the tissue or cell.

In a cell there are numerous organic systems, consisting of oxygen combining-compounds, hydrogen donators, hydrogen acceptors which are produced by metabolism. Among these systems are those which are electromotively active, i. e., they register a potential.

These systems, together with their appropriate enzymes transfer activated oxygen or hydrogen along a chain of reactions which are responsible for the metabolism of the cell. LUND claims to have measured the potentials set up by these processes.

He eliminates other sources of p. d. from such causes as injury and action changes by the use of *unstimulated material* and *symmetrical contacts* (as described above) and believes that he is then measuring the p. d.'s set up by electromotively active compounds, i. e., oxidation-reduction p. d.'s caused by the transfer of electrons, rather than the diffusion p. d.'s of ions produced by the diffusion of the metabolites.

When the oxygen concentration is raised, we know that the rate of oxidation is increased within limits. LUND has also found an increase in potential when the oxygen concentration was changed from a low to a high concentration.

However we noted that oxygen itself interferes with the oxidation-reduction potentials developed at metallic electrodes (See W. M. CLARK 1928). Further experiments are therefore needed to determine whether the p. d.'s so designated are due to *electrons* or *ions*, unless we assume that there is an absence of oxygen inside the cell. BARNES (1939) has shown that frog skin stained by eosin has a higher O_2 consumption than normal skin while at the same time there is a rise in potential. This is reminiscent of the experiments of HEYMANS (1926) on methylene blue who found an increase in metabolism in dogs injected with this dye. LUND has further shown that there exist isolated measurable p. d.'s on *single cells* of a filament (*Pithophora* sp.) and that there was a summation of p. d.'s, so that individual cells have electric polarity. The observed p. d.'s on single cells appear to lend support to the concept of a molecular architecture or mosaic structure of protoplasmic surfaces. His experiments on DOUGLAS fir deal with the problem of the changes in "internal fields" which can be varied by such factors as changes in temperature. Other experiments including those on frog skin, onion roots, *Pithophora* cells and stems show different space-time distributions of p. d.'s. It has been observed that oxidation reactions occur at membranes (GIRARD 1924, 1926, GUNSTALLA 1934 and KROON 1935).

Oxidation-reduction potentials imply spatial movement of negative electrons from oxidant to reductant. If one of these is made up of oriented molecules forming a surface, e. g., oxidant, bathed by a solution containing, or in contact with a similar layer of the partner, e. g., reductant, one may suppose oriented movement of electrons. This implies change of valency for both partners.

The relatively high electrical conductivity of the matrix of the cytoplasm has been assumed on relatively scarce data (e. g., GELFAN 1927). Assuming however that this is true, then the internal phases having oriented layers at their surfaces would produce no perceptible effects outside the cell. The oriented nature of many cells however, together with the well known chemical orientation of morphologically isomorphic cells (e. g., SPEK 1927) may point to different redox p. d.'s. on opposing ends of cells. To complete this picture it is needful to explain the restitution of the original state without annulling the p. d.'s. One may seek an explanation along the lines of the action of oxygen on reduced constituents in an assumed oriented superficial layer, together with several of these molecules *pari passu* with the reduction and oxidation steps. One may suppose that after reversal the electrons are passed on to some constituent of the environment. Alternately, the loss of the oxidant after reduction from the surface into the environment would present another aspect of this picture.

The difference in electrical potential between the inner and the outer sides of the skin of the frog has been the subject of experiments since the work of DU BOIS-REYMOND (1857). PUMPHREY (1934), by the use of polarizing and depolarizing currents, showed that the skin behaved essentially as if it were permeable to K ions in both directions but to sodium or calcium ions in the "polarizing" direction only. PUMPHREY (1935) concluded that the p. d. between the inner and outer surfaces of frog skin behaves like an electrode reversible for K but not for Na or Ca ions. NETTER (1927) obtained a cation series but no anion series by successive contacts of different salts. He interpreted this as meaning that the frog membrane was permeable selectively to cations. Other workers, AMSON (1930), RUBENSTEIN and PEVSNER (1937), LEUTHARDT and ZELLER (1934) believe that there is no correlation between the electrode potential and the permeability of the frog skin.

The presence of oxygen seems to be important in maintaining the level of potential because when N_2 is substituted for O_2 , the potential drops, as shown by LEUTHARDT and ZELLER (1934). TAYLOR (1935) studied the relations between oxygen concentration, oxygen consumption and potential, by the use of flowing solutions. Here again, RINGER solution saturated with oxygen gave higher

values than RINGER solution in air alone. See also KATZIN (1939).

The effects of pH on the fluctuations in potential induced by the Li ion were investigated by TAKENAKA (1936). Agents which lower the surface tension, such as urethanes, were found by DUCE (1937) to lower the potential of the outer surface of the skin but not when in contact with the inner surface.

In a series of papers RIDEAL (1936), FRANCIS and GATTY (1937, 1938), DEAN and GATTY (1937), the effect of various organic substances and of various organic poisons to sustain the potential and respiration of frog skin were studied.

Analysis of the electrolyte content of wet frog skin shows that the Cl present is insufficient to furnish the anion for the univalent ion. (See H. HOAGLAND and RUBIN 1936.) Since we do not know what diffusible ions are present, we cannot be sure how the DONNAN effect would operate. DEAN (1939) interprets the potentials experimentally obtained in frog skin as due to differences in ionic mobilities or the rate of entry of ions into a superficial layer of the skin.

The conclusions of GALEOTTI (1904) and AMBERSON and co-workers (1928, 1929, 1933, 1936) that the basis of the potentials lay in diffusion potentials and semi-permeable nature of living frog skin seem to hold. See reviews by MEYER (1937) and ADAIR (1937).

Other tissues have been used in the study of potential differences. KELLER (1932) used frog and turtle stomach and intestine to show the electro-osmotic processes in resorption of ions; WILBRANDT (1930) the wall of kidney tubules of *Necturus*; GICKLHORN and UMRATH (1928), with vascular plants. They concluded that the p.d.'s are due to HELMHOLTZ layer! LUND (1931) studied polarity potentials in apex of DOUGLAS fir; PUMPHREY (1931) studied the p.d. across the surface bounding the unfertilized egg of brown trout. HÖBER (1905) concluded that muscle is not even permeable to K ion.

FENN and COBB (1934) speak of the high K^+ content of muscle as due to selective permeability for K^+ as compared with other cations and anions; or that K^+ is immobilized in the tissue because it becomes combined with indiffusible anions.

An electrophoretic theory has been used by KELLER (1933, 1934, 1935) to explain the distribution of electrolytes in tissues.

He observed that in a special cataphoresis cell (FÜRTH 1925) some substances, including K salts which are usually found inside cells, move towards the anode, while substances found outside the cells, including Na salts, move toward the cathode. He therefore concludes that the inside of the cells is positive while the tissue spaces are negative. It is difficult to see how these conclusions can be obtained from experiments in which electrodes are merely placed on the outside of organs for comparison with another electrode in the blood.

The concentration of K is invariably greater in the gastric juice than in the plasma, and sometimes the former may be as much as 4 times the latter. The Na concentration is markedly lower in gastric juice than in the plasma of dogs at the time of secretion. INGRAHAM and VISSCHER (1933) suggest that this difference between the behavior of K and Na may mean that K enters the lumen of the gland from the secreting cells themselves, while Na reaches the lumen through the intercellular spaces. This agrees with the well-known fact that K occurs in high concentration within certain cells, whereas Na is very much less abundant.

Conductivity and conductance as a measure of permeability. Measurements of permeability have been attempted by conductivity methods and electrical conductance of suspensions of cells and of tissues. The early literature is reviewed by STILES and JORGENSEN (1914). The electrical resistance of living cells and tissues (PHILLIPPSON 1920); the mathematical treatment of electrical conductivity of colloids and cell suspensions by FRICKE (1924); changes in conductivity of red blood cell suspensions (PONDER and TAYLOR 1925); the electrical conductivity of fertilized and unfertilized eggs (GRAY 1913); electrical conductivity of pure protoplasm (BROOKS 1925); of suspended cells (S. C. BROOKS 1925); the conductance of unicellular organisms (S. C. BROOKS 1922); capacity effects as studied with *Valonia* by BLINKS (1936); COLE (1936) with *Asterias* eggs; the electrical impedance of red blood cells (CURTIS 1936); FRICKE and CURTIS (1935); the effect of alternating currents of long duration and high frequency on electric conductivity and capacity of the blood (McCLENDON 1926), (MORSE and FRICKE 1925); these are only samples of the enormous literature which comprises this subject.

The experiments on conductivity afford a measure of the movement of ions across and around the field of contact. The

experiments on electrical impedance should give us a clearer concept of the thickness of the plasma membrane if the surface boundary of the protoplasm can be so designated. Alternating current at different frequencies has been used for this purpose and definite figures have been obtained. These methods are valuable in conjunction with other chemical means in a better interpretation of permeability.

Permeability H and OH ions. The relative penetrating powers of hydrogen and hydroxyl ions is of considerable importance to the problem of permeability. Many experiments have been done with strong acids and bases to see whether or not penetration into cells takes place. See reviews by HÖBER and HÖBER (1926), GELLHORN (1933) and others. The general conclusion is that strong acids and bases as such do not penetrate, or if at all, very slowly.

JACOBS (1931) and JACOBS and PARPART (1932) have given an excellent analysis of the rôle of the H and OH ions with reference to erythrocytes. According to their semi-quantitative results, they believe that the OH ion and not the H ion is capable of penetrating cells. See also VAN SLYKE, WU and MCLEAN (1923). When electrolytes are used in combination with acids hemolysis proceeds more rapidly. They believe that the reason for this is not due to changes produced upon the plasma membrane, but the results rather argue in favor of a system permeable to OH ions and not to H ions.

It might be commented in this connection that red blood cells are normal only in plasma (excepting oxalated plasma), and any other solution to which they are subjected changes their permeability. This point has also been recognized by JACOBS and PARPART. Therefore the solutions to which these cells were subjected in these experiments were undoubtedly producing injury, so that one could not assume that these results are valid for normal cells. In addition, red blood cells seem to be atypical as compared with many or most other cells in their lipid content. From the works of FRICKE (1926), DANIELLI (1935) and SCHMITT, BEAR and PONDER (1936) it has been suggested that the permeability properties of the cell are determined by a bimolecular layer of lipid, situated either at the surface of the envelope or somewhere within its thickness. In such cases, H ions would have difficulty in penetrating, whereas OH ions could do so easily.

In the case of other cells, as exemplified by *Valonia*, M. M. BROOKS (1923) studied the penetration of strong acids and bases and was able to extract the sap occupying the center of the cell and measure its H-ion concentration colorimetrically during a period of several hours, using of course, different cells for each determination. It was found that, presumably, protoplasm acts as a buffer for the penetrating acid, so that no acid or base could be detected in the sap for a considerable time. In addition to this, the acids were apparently neutralizing various substances in the cell wall or protoplasm (the carbonates or weaker acids of the cell?) so that an increased concentration of CO_2 was produced in the sap. This could be blown off, and the sap always reverted to a pH of 7.0 (the normal pH), as shown by colorimetric test. When all the buffering power of the protoplasm was used up and no more carbonates or bicarbonates (?) were available for neutralizing, then the sap in the cell began to become acid. This might be the mechanism operating in the case of other cells which are not so fortunately arranged as to permit of the isolation and examination of the sap, so that immediate color changes of added or natural indicators would not be noted.

Injury. The detection of injury in a cell is difficult if there is recovery following the injury. Injury usually increases the CO_2 production, and if continued, causes cytolysis of the cell. In most of the experiments with living cells, irreversible injury can be shown by keeping controls and noting the length of life after experimentation. Injury can also be noted in those cells where protoplasmic streaming cannot be observed, but absence of streaming is also compatible with normal life. If injury is not permanent, the streaming is likely to resume after an interval.

When sections of tissues are sliced and not properly treated before use, the effects of injury may alter the experiment. STEWARD's (1937) method of treating slices of storage tissue seems sufficiently adequate to avoid these effects. He allows thin slices to wash in running water so that all waste products of those cells which have been cut are removed before experimenting, while being aerated. He has observed that in the uncut cells, protoplasmic streaming continues during aeration, and the rate of CO_2 production appears normal. This method has been criticized by OSTERHOUT (Nature 1935).

If we interpret as "injury" any condition which is not strictly normal, then increase in rate of accumulation of KCl into the sap

of *Valonia* when concentration of either NaCl or KCl in the external solution was increased, i. e., increase in permeability of the cell, should be called "injury". See BROOKS, S. C. (1929), STEWARD and MARTIN (1934) and STEWARD (1933).

Nearly all experimental procedures which use conditions which differ from the normal may be theoretically said to produce injury. However, only when the advanced stages are noted do we call it so. The border line between increased metabolism which leads to recovery and that which leads to death is not sharply defined.

Visible surface effects. The microinjection experiments of CHAMBERS (1938) and CHAMBERS and KOPAC (1937), and KOPAC and CHAMBERS (1937), in which a droplet of oil was allowed to penetrate into *Arbacia* eggs, are interpreted as indicating that the physical state of the cell surface is essentially that of a liquid rather than of a solid film.

Washing cells to change their surface reactions has been used by many investigators. BRINKMAN and VAN DAM (1920) washed red blood cells free from lecithin and cholesterol and found that their fragility was increased. Artificial analogs have been used to try to explain this process, such as the experiments of CORRAN and McLEWIS (1924), who used solutions of cholesterol and lecithin in olive oil or cottonseed oil and water to change the surface tension and phase reversion. A. R. MOORE (1930, 1932, 1935) placed echinoderm eggs in solutions of non-electrolytes isosmotic with sea water and found that the fertilization membrane was not formed. The eggs subsequently showed, during segmentation, protoplasmic connections between the blastomeres, holding the cells together. This was also noted by CHAMBERS (1938). In *Dendraster* and *Strongylocentrotus* the result is the formation of cell-plates and cell-clusters, but in *Paracentrotus* the cellular bridges are sufficient to hold the blastomeres together closely enough to form complete blastulae. In *Paracentrotus* and *Arbacia* it can be shown that the cohesion of the blastomeres is dependent on the presence of calcium in the surrounding solution, since without calcium the protoplasmic connectives between the cells become attenuated and dissolve. See also HERBST (1900) who found that Ca-free sea water caused the individual cells to drift apart; M. M. MOORE (1932) and GRAY (1923).

An interesting correlation may be made with human red blood cells. When red blood cells are placed in solutions of a

non-electrolyte like glucose, they lose cations. If a small amount of electrolyte like NaCl or KCl is added, this is prevented (See MAIZELS 1937). The reason for the effect of glucose may be cell damage, and may account in part for the results obtained with non-electrolytes and marine eggs.

HEILBRUNN's "Surface Precipitation Reaction" (1930) in which the presence of Ca is essential for the reaction is of interest in this connection, and may be partly explained by the experiments of DANIELLI (1937) who showed that there is an increase in interfacial tension when Ca in alkaline solution is present. The plasmolysis of *Spirogyra* by urea did not occur when Ca had been removed by pretreatment of the cells with oxalate, according to WEBER (1932). The experiments of MAZIA (1937, 1938) and MAZIA and CLARK (1936) showed how Ca can be made to pass from the protoplasm of *Elodea*, where it normally occurs, to the sap where it does not normally occur, by subjecting *Elodea* to a stimulating agent. These experiments all show changes in permeability of the plasma membrane, probably mainly initiated by the presence or absence of cations such as Ca, Sr and Ba, Na, K and Mg. These studies again are based upon the original idea of antagonism of ions from the days of J. LOEB (1922) through the many publications in this field.

The work of COLLANDER (1937) and his colleagues shows that the rates of entrance of representative non-electrolytes into vacuoles or cells of about twenty species of plants are roughly parallel to the partition coefficients between olive oil or ether and water. Improved agreement could possibly be obtained with other oils or mixtures. The linear relation between permeability and partition coefficient between oil and water is well shown by Fig. 17. But among the smaller polar molecules the penetrability exceeds that predicted by the rule just quoted, a fact usually attributed to the presence of aqueous phases in the plasma membrane in addition to oil phases. This conception, the lipid-filter mosaic, has been advanced by COLLANDER (1937). This lipid-filter membrane is much like OVERTON's membrane which contained lipid dispersed in an aqueous layer as mentioned above. From the data given by COLLANDER his conclusion as to the lipid-filter plasma membrane may be accepted with certain possible modifications mentioned below.

From the experiments on non-electrolytes COLLANDER and BÄRLUND (1933) have calculated the permeability of *Chara* by means of an equation

$$d = \frac{D \cdot n}{P}$$

where d is thickness of the membrane, D the diffusion coefficient, n the diffused substance in unit time, and P the permeability. Using the known area of external and vacuolar plasma membranes these membranes can be estimated to have an aggregate thickness of 3×10^{-5} cm, or 0.3μ , provided that there is a continuous oily membrane. This is a microscopically perceptible thickness, and

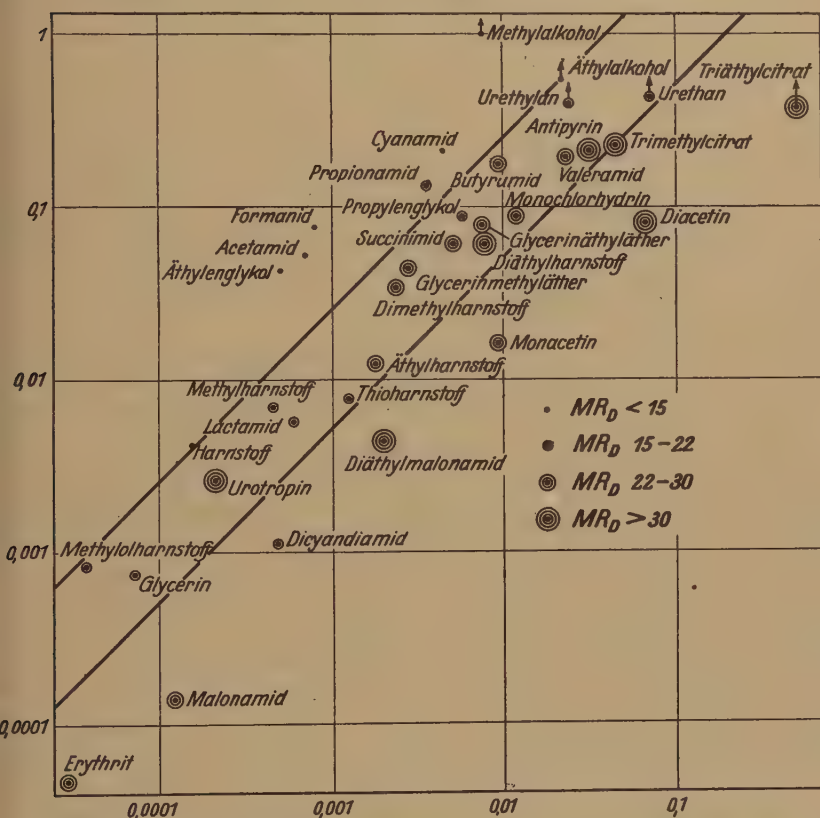


Figure 17. The linear relation between permeability and partition coefficients between oil and water in the case of certain substances. After COLLANDER and BÄRLUND, 1933.

no such visible membrane has ordinarily been detected. A similar thickness has been postulated by OSTERHOUT (1936). COLLANDER has, however, pointed out that a lipid-filter membrane might contain relatively small oil areas which could be supposed to have areas corresponding to the observed permeability.

The supposed existence of continuous films of oil may also be subjected to test by the following methods: Let us imagine that a thin layer of oil is present in or close to the surface of a cell, and that the stability of this film is due to the oil only. The interfacial tension, although low, is still positive. If a break in the plasma membrane occurs, usually due to experiment, the continuity of the film is broken.

In these experiments we can see microscopically the mixing of the aqueous matrix of the cytoplasm with the surrounding medium, and watch the granules and other particles diffuse out of the cell into the medium. If there had been present a continuous oil film, the existing interfacial tension of this supposed film would withdraw the oil from the break and the process would destroy the cell. But actually in many instances no such breakdown occurs, and one sees rather a healing by membrane formation over the opening. Therefore we can only conclude that something other than the oil film forms a new membrane, and the interfacial tensions, although opposing the reconstitution of the plasma membrane, must be not more than of secondary importance.

The experiments of KOPAC and CHAMBERS (1937) on the ingestion of minute drops by *Arbacia* eggs and other cells constitute a further test. An unprotected continuous oil film acting as a plasma membrane would fuse with an adpressed oil globule, so that the oil drop would spread along the surface rather than pass into the cytoplasm as observed. Cells could not be covered with a naked oil film. In the case of vertebrate nerve axons SCHMITT and BEAR (1937) observed that a lipid film exists in the surface. On both surfaces of this film proteins are arranged, and it is apparent that the stability of the thus-formed plasma membrane is primarily due to these layers of proteins. In more nearly normal cells it is rather doubtful whether such an oil layer is present at all, as shown by the same type of experiments. But even if *Arbacia* eggs have such a lipid film with protective adsorbed protein layers on both sides it is still doubtful that an applied oil drop would penetrate through the film at all or would pene-

trate without making contact with the lipid. These facts seem to make the presence of continuous oil films improbable. With this background of permeability to non-electrolytes and these correlated results, we are led to examine the evidence from accumulation of ions — the third type of permeability phenomena.

In this connection, by means of the technique of electroendosmosis, MUDD (1925) and colleagues used various mammalian serous membranes and caused the direct electric current to pass through the membranes. By changing the pH of the external solution he found that the current was reversed at a value between pH 4.3 and 5.3. The conclusions of the writer are that these reversals behave like isoelectric points of proteins, and that therefore the surface is predominately made up of protein.

DZIEMIAN (1939) analyzed the lipid content of a number of red blood cells of various animals and found no correlation between the concentration of lipid in the red blood cell and its permeability to certain lipid-soluble substances whether expressed in terms of lipid per cubic centimeter, lipid per erythrocyte, lipid per square micron of red cell surface, or percentages of cholesterol or phospholipid. These results are contrary to the assumptions of HÖBER (1936) that the red cells of the species more permeable to lipid-soluble substances contain more lipid. He further concluded that it is more probable that a lipid-protein complex rather than a continuous layer of lipid exists.

Summary. To sum up, we can consider the cell as a structure consisting of a plasma membrane which is composed of lipins and different kinds of proteins and amino acids which have different degrees of dissociation, thereby producing areas of molecular or smaller dimensions which are either cation- or anion-permeable. Ion exchange takes place first in the protoplasmic layer or layers, after which vacuoles are affected. The dissociation of the multivalent amino acids or proteins affords a supply of cations and anions for exchange. Metabolism, either aerobic, glycolytic or anaerobic, produces a supply of H ions and either bicarbonate anions or those of organic acids which are available for exchange with cations and anions in the external solution. The mobility of the ions is another factor which is important provided other conditions are equal. K^+ has the highest mobility of cations important in biology. Lipid-soluble areas evidently occur in greater or less degree.

Other factors affecting permeability

A study of the nature of the plasma membrane has many ramifications. These should all be followed in order to obtain a better picture of the problem of permeability. How can we apply the results obtained by means of the x-ray patterns to show us the molecular configuration and position of atoms as shown, for example, by patterns of cell wall (SPONSLER 1928, 1929) of mercerized cellulose (SPONSLER and DORE 1928); of hair, wool and related fibers (WOODS 1938); of protein fibers (ASTBURY 1936); of the hydration and denaturation of proteins (ASTBURY and LOMAX 1935); of *Valonia ventricosa* (PRESTON and ASTBURY 1937) of fibers (HERZOG 1928); of cellulose (FARR and SISSON 1934; FARR and CLARK 1932); of dinitrocellulose (MATTHIEU 1935); of rayon (MATSUMOTO 1934); of chitin (MEYER and PANKOW 1935); of muscle fiber and myosin sols (MURALT and EDSALL 1930); of surface structures (RUPP 1935); of cyclol molecules in protein structure (WRINCH 1936); of hemoglobin and crystalline proteins (WYCKOFF and COREY 1935); of tobacco mosaic (WYCKOFF and COREY 1936); see review by ASTBURY (1938); by nerves (SCHMIDT 1937); by the optical properties of nerve sheaths and axon sheaths; as shown by the work of SCHMITT (1936), SCHMITT and BEAR (1937), BEAR and SCHMITT (1937); the relative concentrations of lipins and proteins in the membrane of different cells, and the effect of this upon permeability of substances?

The proteins of cytoplasm are made up of both ions and molecules. The molecules form a sheet which may be folded up (See WRINCH 1936) with polar side chains on one side and apolar side chains on the other side. The polar side ionizes and attracts and "binds" water, while the apolar does not ionize, and does not attract water. The same considerations apply to starches, glycogen, glyco-, lecitho-, lipo-proteins, lecithin, sterols, fats, etc. These proteins produce the DONNAN equilibrium acting through the plasma membrane (See work on DONNAN Equilibrium by J. LOEB 1921, 1922; TEORELL 1937, MICHAELIS 1925, MEYER, K. H. 1937, Ingraham, LOMBARD and VISSCHER 1933, WARBURG, E. J. l. c.).

The mechanical and physical tests made of protoplasm as shown by surface tension experiments of K. C. COLE (1932, 1937) and HARVEY (1937) and DANIELLI (1937) and its relation to mono-

molecular films of LANGMUIR, GORTER (1937) and MITCHELL (1937) should be considered.

The problem of viscosity of protoplasm is intimately related to that of permeability (HEILBRUNN 1928; HEILBRUNN and DAUGHERTY 1931, 1934). The numerous experiments on action potentials of GASSER, ERLANGER, SCHMITT, BRONK, YOUNG, PUMPHREY and YOUNG (1938) and their colleagues; that on phase boundary potentials as amplified by BEUTNER (1927); the relation between oxidation-reductions and enzyme action; the chemistry of proteins, carbohydrates and the respective metabolism of each; the pH effects on ionization and salt formation should be included. We can merely indicate the relation between these studies and that of permeability.

Conclusion

Future research in Fundamental Biology will be governed more and more by the development of molecular and atomic physics and chemistry. Since Biology is, in its final analysis, organization of matter, only so far as the laws pertaining to this organization are discovered, can we hope to understand its principles. The curtain of darkness which the Sciences of Chemistry and Physics are gradually withdrawing by their discoveries in this foundation of all matter, will be the limiting factor of our understanding.

CHAPTER XII

THE PERMEABILITY OF LIVING CELLS TO DYES

Introduction

The history of the use of dyes begins with the accidental discovery in 1854 of the anilin dye, "mauve", or anilin purple, by WILLIAM PERKIN, a 16-year-old youth, who was trying to synthesize quinine. Shortly afterwards others of the anilin dyes were discovered, such as safranin, methyl violet, eosin, methyl green and methylene blue. Little did WILLIAM PERKIN realize that his discovery was to revolutionize microscopy. BENEKI in 1862 was the first to employ "lilac anilin" in histology. This was the beginning of that vast literature which covers not only the differential staining of tissues, but also the interpretation of the biological significance of the colors produced in both living and dead tissues. (See CONN 1930).

Organic dyes have a very complicated structure, a high molecular weight, and can for the most part be classified as colloids or semicolloids. They are subject to polymerization and tautomerization, and thereby complicate even more the theories concerned with their penetration. They have the property of changing color with change in pH and upon being reduced. Their colors are influenced by the presence of salts producing "salt error" and by proteins producing the so-called "protein error". The latter error, when dealing with living cells, is often not capable of being corrected because of the unknown values involved.

Dyes have been placed by many investigators in a separate category for a very superficial reason — merely because they are colored — and for this reason can be seen inside the cells. A discussion of the permeability of dyes should, however, not be isolated from that of the general subject of permeability of substances. Each dye should be classified under a heading appro-

priate to its properties, rather than grouped in one class because of its possession of color. It is at best an artificial division, which comprises many unrelated species, including non-electrolytes, electrolytes, lipoid-soluble and non-soluble bases and acids, colloids, surface-active and surface-inactive substances and so on. Therefore, in dealing with this chapter the reader is referred to separate chapters comprising discussions on these topics. Theories on the penetration of dyes would necessarily have to be interpreted upon the same basis as those on substances which are similar in chemical or physical constitution.

Since, however, dyes form a separate group by virtue of precedent, they will be treated separately only insofar as the literature is concerned, with the suggestion that they be analyzed into their respective groupings and interpreted in the light of the various properties discussed in the preceding chapters.

Some of the earlier researches did not consider the importance of **purity** of the dye and one cannot, therefore, determine the absolute values of the results. Many of the impure dyes are really compounds of several substances. The fact, therefore, that a color is discerned in the interior of a cell would not prove that the particular dye in question is responsible for the color. Further complications are the absence of agreement among the different manufacturers as to the names of specific chemicals. The latter point has been taken up by a national committee on dyes, which, in conjunction with the National Aniline and Chemical Dye Co., have endeavoured to correct this difficulty. The former point has been illustrated by the work of HOLMES (1926) and HOLMES and FRENCH (1926), SCOTT and FRENCH (1924), HOLMES and SNYDER (1929), HOLMES (1927) and HOLMES and PETERSON (1930) in the study of methylene blue. They found that this dye is oxidized gradually in aqueous solution and rapidly in alkaline solutions from pH 9.0 and higher. The formation of lower homologs occurs which have different dissociation constants and different staining faculties. MACNEAL (1925) extracted as lower homologs from methylene blue, methylene violet and methylene azure A and B. If, therefore, one uses methylene blue containing one of these lower homologs, different results in the penetration of the dye are obtained from those found with the use of the pure dye. SCHULTZ's Farbstofftabellen and the Colour Index of the British Dye Manufacturers are important

in analyzing the composition of dyes under various trade names.

The old idea of allowing, for example, methylene blue to "age" in order to improve its stainability, has been found to be due to the formation of oxidation products which are less dissociated and penetrate more readily. See the experiments of IRWIN (1928) who used very alkaline solutions of methylene blue and obtained different results from those of M. M. BROOKS (1929) in more neutral solutions and purer dye.

The question of impurities in the case of salts is important. Commercial specimens often contain a very large percentage of salts, frequently as much as 20 to 30% of sodium chloride or sulphate arising from the mode of preparation. Therefore, results, obtained by using such dyes in making tests with living cells are not reliable as the effects of the chlorides or sulphates are considerable in themselves and cannot in this case be separated. The double zinc salt of methylene blue, for example, is toxic to cells.

The nature of the aqueous solution of some dyes was studied by ROBINSON (1935). Many of the dyes regularly used in experiments were found to contain foreign electrolytes and have, therefore, been described as colloidal because the solutions contained aggregated particles large enough to be seen in the ultramicroscope. ROBINSON showed this by comparing the properties of very pure dyes with those of impure samples and found them to be quite different. The dyes investigated were all salts of strong acids or bases, namely, benzo-purpurine 4B, bordeaux extra, congo red, congo rubin and methylene blue. He also showed effect of concentration of the dye upon conductivity measurements. This method was used to show whether or not the dyes behaved as ordinary univalent electrolytes or as micelles. If the former were true, then we would expect to obtain a straight line, conductivity decreasing with increasing concentration. The curve actually has a very pronounced maximum in it, with the conductivity increasing at first. This is explained by the formation of micelles having a higher conductivity than the single cations, the micelle formation increasing with increasing concentration. See Fig. 18.

The experiments on the permeability of cells to dyes have grouped themselves around a few theories or combinations of theories which have been in vogue at various times, in which one

property was isolated and to it were attributed the reasons for the penetration of the dye into living cells. As newer methods and knowledge in the field of chemistry have become available, this has been reflected in the experiments on dyes.

Among the earliest of these theories are (1) the classic researches of Overton giving the foundation of the lipid theory which has been in the limelight at various times through three decades with certain changes in its original outline. (2) The acid-base theory of BETHE postulated that the acid-basic nature of the dye and the pH of the interior of the cell were the important factors in determining its penetration. (3) The ultrafilter or pore theory of RUHLAND is based upon the concept that the molecular size of the penetrating substances was responsible for their penetration. (4) Other theories have enlarged upon one or more factors attributable to the study of colloids, such as the importance of the charge on the membrane, adsorption, electrokinetics, phase boundary potentials and e. m. f. and acid-base dissociation. These will be discussed in the above order in the following pages.

In reviewing the earlier work, attention is called to cases in which absence of color in the cell has been interpreted as due to lack of penetration of the dye. This absence of color may have been due to a reduction of the dye. It would have been interesting to ascertain by means of an oxidizing reagent whether this was the case.

It has been further pointed out that in certain cases dyes may be able to penetrate the plasma membrane, and then find nothing in the cell to combine with, and, therefore, not accumu-

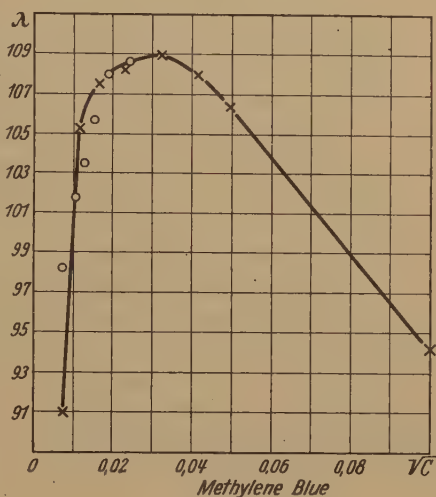


Figure 18.
Relation between conductivity and concentration of methylene blue.

late, although the plasma membrane is freely permeable to them. Or the dye may stain the walls, as in the case of certain plant cells, thereby excluding the view into the interior of the cell.

And finally in comparing experiments the importance of the concentrations used is again emphasized. Different results are obtained with different concentrations, which should not be lumped together as similar merely because the same dye was used. This factor is frequently overlooked.

The lipoid theory of penetration

Since the literature concerning the lipoid theory is so large, only the more recent articles and those which deal with immediate applications to the older theories will be discussed. A great many of these papers have been quoted again and again without critical evaluation. An attempt will be made to interpret them in the light of more recent knowledge. Since many of the dyes used in the earlier work were impure, too much weight cannot be placed on these results.

Among the earliest to study the penetration of dyes into cells were PFEFFER (1886) and EHRLICH (1887). PFEFFER concluded that basic dyes penetrated for the most part while acid dyes did not. The fundamental studies of PFEFFER on the uptake of aniline dyes in plant cells contain many of the problems which are still under investigation with our present-day methods. EHRLICH (1887) used the nervous system of animals and came to the same conclusions as PFEFFER, finding a correlation between lipoid solubility and the ability to stain nerves.

OVERTON (1900) first formulated the "lipoid theory" to explain the solubility of various dyes and other organic substances in cholesterolin and lecithin. He investigated the distribution-coefficient of about 500 substances, both organic and inorganic, between water and lecithin, protagon and cerebrin, using these lipoids either in the form of suspensions or in solution in benzene, and also between water and olive oil, tripalmitin, tristearin, triolein and other lipoids. On the basis of their solubility he formulated his theory.

He concluded that salts of the aniline dyes do not penetrate while bases do; that all those dyes, both acid and basic, which are lipoid-soluble were capable of penetrating living cells, and that, therefore, the plasma membrane was impregnated with a

kind of lipid (lecithin-cholesterol). He states that cells are freely permeable to the lipid-soluble dye base, but not to the relatively lipid-insoluble salts of this base produced by the addition of acids. In acid solution the dye is chiefly in this form, which enters cells with difficulty, while in alkaline solutions it exists largely in the form of readily penetrating free base.

This theory has been accepted and discredited again and again in various forms, and today is the basis of many experiments which have been stimulated by it. For the main part, other combinations of lipid solutions have been used by later investigators, with the idea of improving upon OVERTON'S original concept and bridging over the gaps. These modifications have all been interpreted under "lipoid theory".

A number of exceptions have been found which tend to show that OVERTON'S theory is not of universal application. Most of the basic dyes, i. e., the salts of the dye-bases penetrate easily into cells from dilute aqueous solutions and are accumulated in the sap or the granules; a small fraction of this number is insoluble in a solution of cholesterol in benzol or turpentine, and does not pass from the aqueous phase into the lipid phase. For example, such lipid-insoluble basic dyes as methylene green, methyl green, thionine and methylene azure are able to penetrate living cells and enter the vacuole in plant cells and granules in animal cells, according to RUHLAND (1908), HÖBER (1909), HÖBER and NAST (1913) and GARMUS (1912), while some basic dyes which are of considerable lipid solubility, such as Basle-blue R and BB, nightblue, Victoria blue B and 4 R penetrate with difficulty or not at all, according to RUHLAND (1912). However, these dyes are not very soluble in water, are highly colloidal, and easily flocculated in electrolytes. In spite of this, HÖBER and NAST (1913) and NIRENSTEIN (1920) found that they stain certain animal cells but not plant cells.

By varying the solvent, it was found that more dyes could be included under the lipid theory. A modification of the lipid theory was made by VON MÖLLENDORFF (1918). He used lecithin instead of cholesterol in benzol as the solvent and found fewer exceptions to OVERTON'S theory. On shaking the cholesterol with water and dye, the solution became cloudy and therefore the conclusions are not unequivocal.

VON MÖLLENDORFF (1920) finds that the best basic dyes for staining cell granules are those which are only slightly lipoidsoluble and readily flocculated in contact with acid colloids.

RUHLAND (1912) also found that there were acid-dyes which, though soluble in the aforementioned lipid mixtures, did not penetrate into plant cells. However, in this case also, the dyes are soluble with difficulty in water and highly colloidal and were found by NIRENSTEIN (1920) to stain living *Paramoecium*, and by HÖBER (1909) and SEO (1823) to penetrate into the intestinal epithelium of frog and the blood cells of cow, but that plant cells often fail to show the correspondence expected.

HÖBER (1926) thinks that these exceptions do not disprove OVERTON's theory, but that the vital staining of lipid-insoluble acid dyes is a different process from that of lipid-soluble dyes; that the kind of cell being stained is a limiting factor.

Opalina, and intestinal parasite of frogs, has been extensively used because it takes up dyes readily when fed per os to the frogs and its staining reactions can be followed when changing the pH or other factors of its environment.

HERTZ (1922) found that the entrance of an acid dye into *Opalina* is hindered by narcosis while that of lipid-soluble dyes is not. He also found that he could stain *Opalina* in RINGER solution containing acid dyes which were lipid-insoluble if a slight amount of protein had been added to the solution; whereas lipid-soluble dyes penetrated from a solution of pure RINGER.

NIRENSTEIN (1920), using *Paramoecium*, studied the staining power of over 100 dyes and compared it with the partition coefficients of these dyes between olive oil and water; olive oil plus oleic acid plus water; olive oil plus diamylamine and water. He showed that when oleic acid was added to olive oil more bases took up the dye, although some acid that did not dissolve in oleic acid stained *Paramoecium*. When diamylamine was added to the mixture, there was a parallel between the staining of the artificial system and that of *Paramoecium*. COLLANDER (1921), however, found that 7 of the diamylamine-soluble sulfonic acid dyes are not taken up by plant cells by the plasmolytic method and that the ineffectiveness is due not merely to the failure to combine with cell constituents but to a failure to penetrate except

in minute amounts. His results are also contrary to those of RUHLAND. He comments that RUHLAND used dying material when he obtained universal staining with highly dispersed dyes.

SEO (1923) found that other animal cells behave like *Paramoecium*; that sulfonic acid dyes penetrate red blood cells as a function of solubility in NIRENSTEIN's lipid mixture, but that plant cells often fail to show correspondence.

HÖBER and PUPILLI (1931) have found that those dyes which are insoluble in mixtures of almond oil, oleic acid and diamylamine are as a rule not taken up by red blood cells; if soluble, they are. He concludes that the lipid phase is an appreciable part of the total cell volume of red blood cells.

JURIŠIĆ (1927) studied the uptake of dyes by red blood cells and tried to find a relationship between partition coefficient of dyes and their penetration into red blood cells.

ROBERTSON (1908), using ethyl esters, concluded that the lipid theory does not hold and is not applicable to living tissues. It would appear, however, from his experiments that the organisms used were not alive after being subjected to N/100 HCl or NaOH solutions, and that therefore his tissues were not living when stained. He found that the uptake of certain dyes by ethyl acetate was comparable to that by fat cells of the certain dyes by ethyl acetate was comparable to that by fat cells of the omentum but that this rule did not hold for all types investigated.

The experiments of HANSTEEN-CRANNER (1919, 1924) have been quoted as corroborating evidence of the presence of lipid in the surface membranes of living cells. He exposed plant tissues in single layers to unbalanced salt solutions or distilled water in shallow trays and claimed that fat-like substances derived partly from the cell wall and partly from protoplasmic surface were released. HÖBER (1926) noted that these experiments in which the cells were allowed to remain in distilled water long enough so that their lipoids diffused out, show that the cell is permeated with lipid.

This work has been critically analyzed by STEWART (1929) as to methods used in leaching and in analyzing the solutions. He found that the turbidity of the solutions which HANSTEEN-CRANNER obtained is probably explained by the growth of fungi and bacteria and that the chemical analyses are at fault, so that this observation cannot be used to substantiate the lipid theory.

WULFF (1934), using chrysoidine, which is a vital stain of protoplasm rather than of vacuoles, or inclusions, also believes that its solubility in lipoids is responsible for its ability to stain.

GELLHORN (1928) observed that there was no correlation between lipid solubility and the penetration of acid and basic dyes into frog muscle and frog skin. He also found that acid dyes penetrated frog muscle more readily than basic dyes, and that the reverse was the case when frog skin was used. LIANG (1929) has criticized these experiments on muscle as an unfavorable object for use because the dye can penetrate through the interstices between the muscle fibers rather than through the membrane alone.

LIANG (1929) studied the penetration of dyes into frog kidney and found that only lipid-soluble dyes are secreted from the blood stream by the proximal convoluted tubule while lipid-insoluble dyes can enter only through the glomeruli. The secretory ability of the tubules can be diminished reversibly by narcotics or asphyxia. Lipoid-insoluble substances, such as dioxyacetone can, by virtue of their small molecular structure, be secreted by the tubule. The same is true for K and Rb. On the other hand, Ca and Mg and the anions, such as I, Cl, SO_4 and PO_4 , and glucose, which has a large molecular configuration, cannot be secreted from the blood stream by these tubules. Lipid-insoluble sulfonic acid dyes can penetrate only from the artery into the uriniferous tubules and become only slightly concentrated there, while lipid-soluble dyes become considerably concentrated. The question of dye penetration in the case of kidney structure is complicated by the additional factor of secretion.

BEUTNER (1930, 1931, 1932) and his collaborators in a series of experiments with living cells and non-living models also believes that stainability and permeability of dyes to cells are explained on the basis of partition coefficients and electromotive force of the system. His model consists of a series of solutions, for example, as follows:

— saline/solvent mixture with quinine/solvent mixture with oleic acid/saline.

He found, for example, in the case of white cells (BEUTNER, LOZNER and CAYWOOD 1931) that a dye which was taken up exclusively or preferably by a non-aqueous solution of oleic

acid, produced a differential stain on the nucleus of white blood cells. Also the reverse was true. Any dye with a preference for a quinine-containing mixture preferably stains the cytoplasm exclusive of granules, and leaves the nucleus unstained. The authors conclude that the staining of the non-aqueous solutions runs parallel to the staining of tissues; that electromotive forces, due to the different composition of adjacent parts of the cell, are produced; that it is not the basic or acid character in aqueous solution which is important, but whether they are attracted by such a water-insoluble acid as oleic acid in non-aqueous solution.

BEUTNER assumes that there is no difference in potential between the two solutions, oleic acid and the quinine solution in his model, and that there is no diffusion potential between these two. This assumption is open to question.

OSTERHOUT (1929—1931) has also produced a model which he believes shows the manner of penetration of substances into protoplasm. This is dependent upon the theory of partition coefficients and is more fully discussed in Chapter XI.

IRWIN (1927) has attempted to show a correlation between uptake of dye by chloroform with that taken up by plant cells. In these papers the lipoid theory has been amended from simple partition coefficient to one of multiple partition coefficients. When a larger number of dyes is applied this theory cannot be validated (See BROOKS and BROOKS, 1932) and is theoretically unattainable. In an earlier paper (1926) IRWIN mentions that partition coefficients are responsible for entrance or exit of the dye, but proves nothing experimentally. See HOFFMAN (1927) who has commented on this.

MCCUTCHEON and LUCKÉ (1924) tried to obtain some relation between the partition coefficient of oil of sweet almonds and water in the case of brilliant cresyl blue and NH_4OH and NaOH . This experiment has been commented on by IRWIN (1925) that even if there is some relation between these various phases and dyes placed in them "it may not necessarily prove to be the case with living cells". This criticism applies equally to the above experiments of IRWIN with artificial systems using chloroform and multiple coefficients.

It has been pointed out by BROOKS and BROOKS (1932) that most of the work on partition coefficients which has been tested with dyes does not distinguish between solubility of dry dye in

lipoid phase and solubility of aqueous solution of dye in contact with lipoid phase. In the latter case part of the water dissolves in lipoids and one obtains a mixture of lipoid and water and dye. Therefore, it is not justifiable to speak of partition coefficients of dyes between lipoid and aqueous phases when there is really present a mixture of these three. For this reason, partition coefficients as determined in this manner do not have an exact quantitative value. Furthermore, BÄRLUND (1929) has pointed out the indefinite conception attached to this idea, because one does not know whether the solubility properties of the various lipoids agree or not.

To illustrate this point, according to COHEN and PREISLER (1930), dry brilliant cresyl blue dissolves in CHCl_3 as such, but if water is added to the dye before it is shaken up with CHCl_3 it breaks down to form lower homologs and one does not obtain the partition coefficient of the dye itself but that of a few of its oxidation products.

SCARTH (1926) on the other hand, has suggested that the colloidal substance in the sap as well as in the cytoplasm which has an affinity for dyes, behaves as an ampholyte and that it is partly hydrated lipoid enveloped by a mono- or bimolecular film which must be considered in evaluating theories. STEARN and STEARN (1930) are in agreement with the importance of the amphoteric nature of the membrane, but consider it composed at least partly of protein, while WOODHOUSE and POCKWORTH (1932) believe that the protein as well as the lipoid envelope exerts a selective permeability. They examined a number of water-soluble dyes and noted the distribution between corpuscles and the liquid phase after 30 minutes, comparing these results with those obtained with egg white used in place of the erythrocytes. They found a parallel which led to these conclusions.

The experiments on microinjection of CHAMBERS and KOPAC (1937) in which a small globule of oil was pushed into an *Arbacia* egg, shows that there must be other properties beside lipoid constituting the plasma membrane, otherwise the oil globule would have coalesced with it instead of proceeding into the interior of the egg.

The ultra-filter theory, dispersion and adsorption in relation to dyes. Since the ultra-filter theory implies the presence of capillaries and the movement of particles through them, all the

properties dealing with electrokinetics, including phase boundary potentials, charge on the membrane, dielectric constants, charge on the dye particle, size of the dye particle with respect to size of the capillary and their effects on permeability have been considered by various writers in the study of penetration of substances. See Monograph by KELLER (1932) and PFEIFFER (1927).

The ultra-filter theory of the diffusibility of dyes was based by RUHLAND (1912) on the concept that their permeability depended upon the degree of dispersion of the dye particle. He allowed dyestuffs to ascend with the transpiration current and subsequently filter through the tissue. The rate at which they appeared in the cells followed approximately the order of their rate of diffusion through filter paper or their diffusibility in gelatine, so that in the case of *Beggiatoa*, the molecular volume was evidently the principal property which determined the ratio of penetration. COLLANDER (1921) commented that the plant *Beggiatoa*, with which RUHLAND worked, was probably injured, judging by the size of the molecules which were admitted.

The ultra-filter or pore theory leaves out of consideration the numerous experiments with uncolored molecular disperse substances which are not in agreement with the theory. It also does not account for the experiments which COLLANDER (1921) has performed on numerous highly dispersed acid dyes which do not color the cells. In other experiments other properties enter into consideration. For example, basic dyes penetrate living cells better than acid dyes; some lipoid-soluble dyes penetrate better than lipoid-insoluble ones; differences in rates of penetration are found between plant and animal cells. Therefore the results found with *Beggiatoa* could not be considered of general application.

The experiments in which ROHDE (1920) fed boric acid or Na carbonate to frogs would tend to discount this theory since the molecular dispersion of these dyes is increased by this treatment, and yet the highly colloidal dyes which caused death in the controls on injection passed rapidly out of the kidneys without injuring them.

SCHÖNFELDER (1931) used a number of organic substances of different character in the study of their permeability into *Beggiatoa mirabilis* and found that lipoid-soluble surface-active substances of large molecular volume penetrated faster than lipoid-insoluble

surface-inactive substances of the same molecular volume, thus showing the importance of considering more than one factor. With small and intermediate molecules, the influence of lipoid-solubility is small and hardly noticeable.

KOPACZEWSKI (1928) using petals of white flowers and *Paramoecium* noted that only acid dyes penetrate the petals and the degree of penetration depended upon the molecular dispersion of the dye; whereas, in the case of *Paramoecium*, basic dyes penetrated and there was no such relationship as above. Colloids and molecular-disperse dyes penetrated with equal facility.

Table XXXIII from the work of NISTLER (1931) shows the changes in molecular dispersion with changes in concentration of various dyes. He used the diffusion microscope for measuring the rate of diffusion of dyes. He showed that the rate of diffusion in water depended upon the concentration of dye. The more concentrated the dye, the greater was the diffusion coefficient in general, with several exceptions in which there is no difference and in several cases where the coefficient is smaller with higher concentrations.

Others have attempted to use the protective ability of colloids in connection with the penetration of dyes. LIESEGANG (1929) used gum arabic with trypanflavine and found a slower diffusion through 5% gelatine than in the controls; and BECHHOLD (1908) showed that methylene blue was prevented from passing through an ultra-filter by the addition of egg albumen to the solution. HERTZ (1922) added extracts of various tissues to the external solution in which *Opalina* were placed and found that they were more readily stained with insoluble lipoid dyes. PLATTNER (1924) found that indigo-carmin RINGER solution which was free of albumen did not stain the bile capillaries under certain imposed conditions, whereas when albumen was added staining occurred. BENNHOLD (1927) noted a more rapid diffusion of brilliant congo red when serum was added to a 5% gelatine gel. If unstable hydrophobic colloidal stains are dissolved in a solution of serum albumen, this acts as a protective colloid (KISZELY, 1935).

The effect of the presence of proteins may be either an effect on the membrane, in the case of living cells, or may be due to a combination of the protein with a portion of the dye, which would then be taken out of active participation, thus leaving the rest in solution. In this way the aggregation of dye would be more dilute

Table XXXIII. Diffusionskoeffizienten ($D \cdot 10^6$)
 von gemessenen Farbstoffen in Abhängigkeit von der Konzentration

Nr.	Name	Anmerkung	0.125 %	0.062 %	0.031 %	0.016 %	0.008 %	0.004 %	0.002 %
1	Alkaliblau	. . . frisch . . .	0.318	0.325	—	—	—	—	—
1a	Alkaliblau	. . . 2 Monate . .	0.399	0.404	—	—	—	—	—
2	Aurantia	. . . frisch . . .	1.7	2.6	2.9	3.5	4.4	—	—
2a	Aurantia	. . . bis 1 Tag . .	4.05	3.8	4.1	4.25	4.6	—	—
2b	Aurantia	. . . 1 Jahr . . .	5.1	5.7	6.2	6.8	7.5	—	—
3	Berlinerblau	. . . wasserlösl.	0.176	0.176	0.176	—	—	—	—
4	Bismarckbraun	3.04	3.54	3.8	5.1	5.6	6.7	—
5	Cyanol	1.4	1.9	2.3	2.4	2.5	—	—
6	Eosin A	. . . frisch . . .	3.89	3.78	3.81	4.24	—	4.45	—
6a	Eosin A	. . . bis 1 Tag . .	2.15	3.12	3.10	—	3.32	—	4.11
7	Erythrosin	. . . frisch . . .	2.91	3.25	3.13	3.28	3.35	3.18	—
7a	Erythrosin	. . . einige Std.	3.18	3.59	3.54	3.78	—	4.05	—
7b	Erythrosin	. . . einige Tage	3.28	3.52	3.69	4.01	—	4.51	—
7c	Erythrosin	. . . 2 Monate . .	3.65	4.09	4.09	4.14	—	4.64	—
7d	Erythrosin	. . . gesamt . . .	3.25	3.61	3.61	3.8	—	4.1	—
8	Isamurblau	0.98	1.2	1.54	1.6	1.7	—	—
9	Kongorot	1.1	1.0	0.94	1.06	1.1	1.31	1.29
10	Kongorubin	1.2	1.65	1.86	2.05	2.1	2.4	2.45
11	Magdalarot	2.24	2.36	2.63	2.87	2.92	3.43	3.68
12	Malachitgrün	. . . 1 Jahr . . .	2.3	2.52	2.94	3.2	—	3.88	—
13	Methylblau	. . . bis 5 Tage	0.42	0.71	1.01	0.96	—	—	—
13a	Methylblau	. . . 1 Jahr . . .	0.69	1.21	1.39	—	—	—	—
14	Methylenblau	1.47	2.18	2.93	3.74	4.36	4.91	5.72
15	Methylgrün	. . . frisch . . .	3.46	5.45	7.55	—	—	—	—
15a	Methylgrün	. . . 1 Jahr . . .	7.48	7.5	8.5	—	—	—	—
16	Methylviolett	3.2	4.0	4.24	4.4	4.47	4.74	4.76
17	Neutralrot	. . . 1 Tag . . .	2.7	3.34	3.5	3.8	3.7	3.76	—
17a	Neutralrot	. . . 1 Jahr . . .	3.68	3.9	4.3	4.6	—	5.5	—
18	Ponceau	3.9	4.2	4.35	4.38	4.4	4.7	4.9
19	Pyrrholblau	. . . bis 17 Std.	0.5	0.86	1.08	1.26	—	1.14	—
19a	Pyrrholblau	. . . 16 Tage . .	0.22	0.52	0.76	0.98	1.13	—	—
20	Rose bengale	1.83	2.83	3.14	3.6	3.9	4.07	4.1
21	Rubin S	3.85	3.41	3.49	4.05	4.09	4.13	4.4
22	Rutheniumrot	—	—	—	—	—	2.76	3.63
23	Safranin	3.43	3.71	3.9	4.07	4.56	—	—
24	Säurefuchsin	3.81	3.9	4.07	4.15	4.19	4.21	4.41
25	Trypaflavin	5.74	5.96	5.93	—	5.1	—	5.63
26	Uranin	. . . bis 7 Std.	7.93	6.23	5.48	5.75	5.03	—	4.89
26a	Uranin	. . . 11 Tage . .	2.14	3.5	4.12	4.19	4.58	—	—
26b	Uranin	. . . 2 Monate . .	2.3	3.08	3.83	3.95	4.24	4.7	—
26c	Uranin	. . . gesamt . . .	3.75	4.1	4.43	4.65	4.65	4.7	4.89
27	Wasserblau	1.88	1.55	1.67	1.66	—	1.71	—

and; therefore, more molecular-disperse and the dye could diffuse faster and consequently penetrate more readily. This may account for some of the results obtained, in which the addition of protein favors penetration.

According to the **adsorption theory**, the higher valency, the greater adsorption of the substance. However, this has not been found in MANN's (1924) experiments. Methylene blue, according to PFEFFER (l. c.) is taken up almost completely by the vacuole, and the protoplasm is not stained. If this dye is adsorbed one would expect that it would be taken up by the highly colloidal protoplasm rather than by the watery contents of the vacuole. Of course this is a basic dye and adsorption on protoplasm takes place presumably only with acid dyes on account of the charge on the membrane. PFEIFFER (1927) states that the theory does not hold in every case, citing examples where structures are colored in the absence of colloids. GORTNER (1927) studied the nature of the combination between certain acid dyes and proteins and concluded that it was due to adsorption.

PISCHINGER (1927) measured the rate of diffusion and the concentration of methylene blue diffusing through varying concentrations of gelatine and through water alone at various H-ion concentrations. He found that the diffusion through gelatine increased as the H-ion concentration increased and decreased as the concentration of the gelatine decreased, whereas the H-ion concentration had no effect upon the rate of diffusion through water alone. When an acid dye was used, such as Kristallponceau, the rate of diffusion through gelatine decreased as the H-ion concentration increased, and as in the case of methylene blue, decreased as the concentration of gelatine increased. He interprets these results as follows: that vital staining depends upon the relation between the charge on the membrane and the dye, and that adsorption enters into the discussion. He also experimented upon the precipitation effect between the dye and egg albumen and found that this depended upon the relation between the charge on the dye and on the albumen.

KREBS and NACHMANSOHN (1927) studied the curves for adsorption by kaolin of 20 different acid stains and measured them colorimetrically, and concluded that non-specific surface properties are chiefly responsible for staining. They compared these results with those found with *Paramoecium* and noted that the

acid dyes which were not adsorbed by kaolin also did not stain living *Paramoecium*.

REDFERN (1922) used discs of carrot root and placed them in solutions of basic dyes and noted the concentration inside became greater than that outside, and suggested that since the logarithm of the external concentration plotted against the log of the internal concentration at equilibrium was a straight line, that this was not proof but evidence that the process was one of adsorption.

The adherents of this theory stress the importance of the electric charge as determining penetration. If a trace of a polyvalent ion is added, the charge can be reversed. SCARTH (1923) states that the cell wall is stained by methylene blue because of the charge on the surface and that the presence of trivalent salts or H^+ in the external solution reverses the charge on the wall or on the protoplasm. SEKI (1934) also states that the charge on dyes can be reversed from negative to positive by the addition of salts of Al or Cu.

BENOIST, GOLBLIN and KOPACZEWSKI (1929) determined the charge on a number of dyes and noted that plant cells (white flower petals) were colored mainly by acid dyes and animal cells by basic dyes. This is contrary to HÖBER, who states that only basic dyes can penetrate living cells. They state that electrophoresis is the only method for determining whether the charge is positive or negative. They determined the degree of dispersion by dialysis. FÜRTH (1925, 1927, 1929) has measured the electrophoresis of various dyes in aqueous solution and as influenced by salts.

PISCHINGER (1927) used methylene blue as a basic dye and crystal ponceau, allowing them to diffuse into gelatine and determined the rate of diffusion colorimetrically. He thinks that the determining factor is the difference in charge between dye and protein, and that histological staining depends therefore on the amphoteric nature of tissue colloids. RUNNSTRÖM (1928) states that the ability of a dye to be adsorbed by a colloid depends upon the pH.

The whole subject of adsorption and protection of colloids against precipitation is briefly mentioned here. An extensive literature can be found in W. OSTWALD's works, ABDERHALDEN's Handb. der biol. Arbeitsmethoden and others.

TRAUBE and KÖHLER (1915) have suggested a surface-active theory in which a parallel between permeability to a dye and its surface tension in an aqueous solution was determined. In the case of albumen, basic dyes are more strongly adsorbed than acid dyes, but this can be explained by consideration of the electric charge involved.

In the case of cells, most of the bodies stained are colloids with negative charges. This reason has been ascribed to the ready adsorption of electro-positive dyes, such as are included among the basic dyes. When an acid dye is adsorbed, the whole molecule is taken up; when a basic dye is adsorbed, only the positive colored ion is taken up (FREUNDLICH and LOSEV, 1907). PANTANELLI (1915) states that only the ions are adsorbed.

Purely physical experiments have been devised by ARCISZEWSKI, CZARNECKI, KOPACZEWSKI and SZUKIEWICZ (1928) to study the capillarity of three types of dyes, electro-negative, electro-positive and amphoteric, by determining the height attained by the dye on strips of filter paper at the end of 24 hours. They used four methods: dilution, dialysis with collodion sacs, changes in viscosity, and surface tension. The role of concentration appears of prime importance, but other factors are also to be considered. The effect on capillarity of the charge of the walls which may be reversed by acids or positive colloids is commented upon. These experiments cannot be interpreted in the light of rate or equilibrium, but are of comparative value.

It has been concluded by KOPACZEWSKI and ROSNOWSKI (1928) in a study of the effects of various reagents on the electro-capillarity of certain dyes rising in filter paper, that more regular results are obtained when equiionic rather than equimolecular concentrations are used. A wide variety of solutions were used including monovalent and bivalent organic and inorganic salts of sodium, acids, bases and various chlorides of different valencies. The usual effects of cations and anions on colloidal behavior was found, namely, that cations favor the penetration of positive colloids, and flocculate the negative colloids, and that anions have the inverse action. The authors believe that the irregularities of the anions and cations which exist under the name of the HOFMEISTER Series, may be overcome in part by the use of equiionic rather than equimolecular concentrations; and that the

special position occupied by certain ions, such as the H ion or the OH ion may be attributed to their mobility.

BENOIST, GOLBLIN and KOPACZEWSKI (1929) studied the molecular dispersion of a number of dyes and their electric charge as related to living cells. They concluded that plant cells allow acid dyes of a large degree of dispersion to penetrate, while animal cells are colored exclusively by the positively charged dyes without regard to their degree of dispersion.

MICHAELIS (1925) has perfected semi-permeable collodion membranes and noted that some can be made cation-permeable and others anion-permeable. The general cause of retardation of ionic mobility within the membrane may be supposed to be increased friction of the water envelope dragged along by the ion in the capillary canals of the membrane. The difference of the effect on the cations and on the anions may be attributed to the electric charge of the walls of the canals.

REISS (1932) changed the permeability relations of a collodion membrane to oxidation-reduction dyes. The membrane was permeable to water and to a large number of redox dyes. If the membrane was first dyed with one of the redox dyes and then placed between two solutions, one of which contained a reducing agent, the membrane remained impermeable to the reducing agent as long as it was stained. As soon as the reductant reduced the color of the membrane, however, equilibrium was quickly established between the solutions bathing the two sides of the membrane. Presumably the charge on the membrane was changed by the oxidized dye preventing passage of the reducing agent. This experiment resembles those of MOND and HOFFMAN (1928) in which the charge on the membrane was changed by rhodamin so that it became anion-permeable instead of cation-permeable.

PICK (1935) injected various fluorescent dyes into frogs and then made histological examinations of the areas stained in the tissues. A crude attempt was made to ascertain whether the dyes were negatively or positively charged.

The elimination of dye by the stomach and pancreas after intravenous injection has been studied more recently by KOBAYASHI (1926), DAWSON and IVY (1925) and CRANDALL, OLDBERG and IVY (1929). INGRAHAM and VISSCHER (1935) made a careful study of the discrepancies in the results of the former investigators and found that no electro-negative dyes appear in the gastric juice and

no electro-positive dyes appear in the pancreatic juice. The earlier investigators overlooked certain properties of dyes which made the results appear contrary to those expected. However, it was pointed out that some of these dyes appeared in the reduced or leuco form and the effects of others could be explained because they were found to be amphoteric or partially amphoteric, as judged by cataphoretic determinations. In parallel experiments on the passage of dyestuffs through collodion membranes, they found that a charged membrane can completely restrain passage of dyes of opposite charge and attributed this to polar adsorption. The effect may depend upon the size of the pores. Various conditions may arise to give different results. If a pore is large, the resulting field through the center of the pore may be sufficiently weak to allow an ion of like charge through. In the same pore an ion of opposite charge would become adsorbed or bound to the membrane-ion, thus blocking the passage. If the pore is of small diameter and charged, the electrostatic field in the center of the pore may be so great as to prevent the passage of an ion of the same charge. Thus, depending upon the size of the pores a membrane may stop large ions of opposite charge and pass those of like charge as in INGRAHAM and VISSCHER's experiments with ordinary collodion or tanned gelatine membranes, or it may stop small ions of like charge and pass those of opposite charge as in the case of the "dried" collodion membranes of MICHAELIS (1925). Recognition of these differences may account in part for the discrepancies found in various types of experiments with living cells and dyes.

Acids and bases in relation to permeability to dyes

One of the theories which has received a great deal of attention concerns the effects of acids and bases in relation to the penetration of dyes. The earlier workers considered the pH of the external solution as the important factor, while later the pH of the interior of the cell was also included. In addition to this, KELLER (1932) in an extensive monograph, believes that there is a relation between the acid or basic nature of the solution to the charge on the membrane, the dielectric constant, and phase boundary potentials, whereas BEUTNER (1932) considers that the acid-base properties of the solution are not concerned with stainability.

HENNEGUY (1896) first made the observation that acid dyes stain in an acid medium and basic dyes in a neutral or an alkaline

medium. OVERTON (1900) first supposed that only the free dye base of the basic dyes entered cells and then abandoned this idea. HARVEY (1911) came to the conclusion that the first hypothesis was correct. However, BETHE (1909 to 1922) followed by ROHDE (1917, 1920), were the first protagonists of the acid-base theory. They observed that acid dyes penetrate better from acid solutions and basic dyes from alkaline solutions; and that acid dyes are taken up by cells having an acid reaction, and basic dyes by cells having a basic internal reaction. They attributed the penetration of these dyes to the effects of pH on the dye and on the plasma membrane. Bethe concluded that protoplasm resembled the ampholyte character of certain substances like albumen and gelatine by which the dye-anion or the dye-cation combines with bases or acids to form salts. Penetration was supposed to be dependent upon the pH of the external solution and upon the pH of the cell itself. When cells have a sufficiently acid reaction, staining with acid dyes should occur more readily than with basic dyes. See also POHLE (1924) and HENZE (1912). The latter states that the blood cells of ascidians are strongly acid; that the body cells take up the basic dye, methylene blue, but the blood cells become only faintly blue, while taking up such acid dyes as cyanol or eriocyanin.

BETHE (1922) also used parchment paper tubes and notes that acid dyes in an acid reaction stain the membrane much deeper and the dye penetrates to the inside faster. HÖBER (1926) points out that all these observations can be explained according to an observation which OVERTON (1899) first made, namely, that the addition of acid to an acid dye or of alkali to a basic dye and subsequent shaking with a lipid solution changes the partition coefficient of lipid to water. The addition of acid to an acid dye causes the formation of free acid dye, while the addition of base to a basic dye causes the formation of free base, and these are as a rule more lipid-soluble than the salts. (See also ROBERTSON 1908.)

Since acids repress the dissociation of acid dyes and bases that of basic dyes, there arises a greater concentration of undissociated molecules. These penetrate in general according to the laws of diffusion so that BETHE's theory would make the lipid theory unnecessary.

The case of red blood cells is interesting inasmuch as they are supposed to be anion-permeable rather than cation-permeable,

as has been found for other cells and tissues in general. For this reason, the results with basic and acid dyes should be opposite to those found for other tissues. This has been found to be the case.

Effects of changing pH values: Various attempts have been made to change the pH of the inside of the cell. Among the earliest are BETHE (1909), WARBURG (1910) and ENDLER (1912b) with *Spirogyra* cells. HARVEY (1914) stained *Paramoecium* and *Elodea* with neutral red and then immersed the organisms in solutions containing either a strongly dissociated or weakly dissociated base. He found that the color inside the cells was not affected when the organisms were placed in solutions of strong base, but that injurious effects were noted, such as stoppage of ciliary movement and cytolysis of *Paramoecium*, even before any appreciable amount of the NaOH had penetrated. (When placed in solutions of the weak electrolyte NH_4OH , the color inside was changed very rapidly, showing a rapid penetration of the undissociated molecule, but there was no effect upon the cell activity.)

WIECHMANN (1922) changed the reaction of blood cells with CO_2 , which is known to penetrate readily into cells (ENDLER 1912, JACOBS 1920, M. M. BROOKS 1923) and found that no acid dyes were taken up by observing the dye concentration of the external solution.

Following the same methods of changing the pH of the inside of the cell by using weak acids or bases (See also JACOBS 1920, 1922), McCUTCHEON and LUCKÉ (1924) noted the effects on the penetration of the basic dyes, brilliant cresyl blue or neutral red, into *Nitella*, *Gonionemus* and *Asterias forbesii* eggs. They found that the dye penetrated less readily if the reaction inside was made alkaline and more readily if it was made acid. Since proteins combine readily with basic and not with acid dyes in relatively alkaline solutions and show the reverse behavior in more acid solutions, the state of the dye in the external solution would be of less importance than the condition of the protein in the cell. This is the theory which McCUTCHEON and LUCKÉ (1924) tested in their experiments. They concluded that the dye under normal conditions was able to accumulate by combining with some acid constituent of the protoplasm. Although no acid dyes were used in their experiments their conclusions seem to be in harmony with STEARN and STEARN'S (1931) concept of the amphoteric nature of

protoplasm and that of ZIPF (1929), who interprets staining as chemical combination. STRELNIKOV (1924a) also observed accumulation of the basic dyes chrysoidin, brilliant cresyl blue, neutral red and methylene blue by *Paramoecium* in concentrations indicating more than 25,000 times that found in external medium.

Much of the work purporting to have changed the pH of the inside of the cell is subject to revision or amendment. If the inner reaction of the cell implies the reaction of the protoplasm, then it is not possible to discuss the theoretical aspects of this theory, inasmuch as the reaction of the protoplasm in most cases is not known. (CHAMBERS and POLLACK 1927.) If the inner reaction of the cell implies the pH of the various parts of the cell, such as sap, cellular inclusions, color of petals, there is evidence both for and against the theory. And lastly, most of the experiments reported make no distinction between rate of penetration and final concentration of the penetrating dye at the end of the experiment.

Many investigators assume because some visible change in pH of some constituent of the cell has taken place, that this implies a change in the reaction of the protoplasm. The protoplasm is a highly buffered substance (See CHAMBERS and POLLACK 1927) and for this reason we much distinguish between reactions which occur when a change in color takes place in the plastids, for example, which contain natural color, or in the chromatophores, or in some artificially colored inclusions (such as in the experiments of HARVEY 1911, PANTIN 1923, BETHE 1909, CHAMBERS 1928) and color changes in the protoplasm itself. CHAMBERS and POLLACK demonstrated the buffer action of living protoplasm. They were unable to change the color of the protoplasm of starfish eggs which had been stained with an injected solution of an indicator dye and then placed in solutions containing NH_3 or CO_2 which are known to penetrate readily. (See JACOBS 1920.) Some investigators use color changes in the sap to show permeability in such cells as *Nitella* and *Valonia*. (HOAGLAND and DAVIS, BROOKS, M. M., l. c.) These are valid only in showing that substances must have gone through the protoplasm in order to penetrate into the sap without necessarily changing the pH of the protoplasm.

BETHE used neutral red as a stain to determine the reaction of the cell. This stain does not color the protoplasm but only the inclusions so that the reaction of the cell was not determined.

Although it has been shown that certain results, which have been religiously quoted as substantiating BETHE's theory of the dependence of the reaction of the cell upon penetration of dyes, really do not have any bearing on this subject, nevertheless there are a great many interesting results which can be used to build up the theory of the dependence of the reaction of the external medium upon the ability of the dye to penetrate. In this connection, the animal parasite, *Opalina*, which grows in the intestine of frog, has been extensively used with varying results.

ROHDE (1917) found that *Opalina*, which grows normally in an alkaline medium, takes up only basic dyes, while *Balantidium*, which is found normally only in weakly acid medium, takes up only acid dyes. RUMJANTZEW and KEDROWSKY (1926) were not able to confirm this finding for examples of the second group of acid dyes for *Opalina*. They found that vital staining with basic dyes caused no diffuse coloring but produced aggregation of the dye in the granules and vacuoles. The intensity of the staining with basic dyes was greater in alkaline solutions than in acid. They also used two other Protista, *Chlamydomorphys major* from the intestine of horse, which is alkaline in reaction, and several Protista from boggy waters having a pH of 4.0. Apparently in this case the normal reaction of the environment had no effect upon the uptake of dyes, for neither of these Protista were able to take up acid dyes, but both took up basic dyes. It should be again noted that the internal reaction of living protoplasm is difficult to measure and that in the majority of the references the experiments purporting to have obtained the pH of the protoplasm, the methods are open to criticism. (See COLLANDER 1921.) It is not possible to crush tissues into a pulp, add indicator, and proclaim the reaction of the mass of destroyed, abnormal juice as the pH of normal protoplasm. (ROHDE 1917.)

RUMJANTZEW and KEDROWSKY (1926) have attempted to obtain the pH of the interior of *Opalina* by crushing and grinding the animals and adding indicator to the extract. This is obviously not the normal pH of the animals. They obtained a pH of 6.1 to 6.4 which agreed with that found by CROZIER (1923). CROZIER placed *Paramoecium*, *Opalina* and insects in solutions of indicators (acid-base) until the indicator penetrated and concluded that the reaction of protoplasm was acid by noting the color found inside.

Since the ordinary H-ion indicators do not penetrate normal cells (See SCARTH 1924, KÜSTER 1926, RUMJANTZEW and KEDROWSKY 1926, FLOWE 1931) the tissues must have been injured if dyes penetrated. Injury produces increased acidity, so that this is probably why CROZIER found the protoplasm of these animals acid. Even when microinjecting such indicators into living cells, great care must be taken not only in the method of injecting, but also in reading the true pH after injection (See CHAMBERS 1927) since slight injury and consequent production of acid give erroneous values. The fact that the pH found by RUMJANTZEW and KEDROWSKY (1926) was coincident with that found by CROZIER (1923) for *Opalina* confirms the conclusion that in both cases the animals were injured.

BODINE (1927) attempted to measure the pH of the protoplasm of *Fundulus heteroclitus* by placing micro hydrogen electrodes into the egg. The readings were taken instantaneously as soon as the cell had been cut and required not more than a few seconds for completion. In view of the findings of CHAMBERS (l. c.) on methods of micromanipulation, which showed that an injury in the cell resulted in an immediate release of acidity, one wonders if BODINE had allowed his electrodes to remain in the cytoplasm for a time and then taken a reading, whether he would have obtained the same value. CHAMBERS found that a time interval was necessary after injecting an acid-base indicator before taking pH readings in order to allow the dissipation of the excess acidity which resulted in the necessary injury involved in getting the indicator into the cell.

The experiments of McCUTCHEON and LUCKÉ (l. c.) attempted to change the H-ion concentration of the interior of starfish eggs, *Nitella* and *Gonionemus* to see what the effect would be on the penetration of the dye. In considering these experiments from the present day point of view, one must conclude that they evidently did not change the pH of the protoplasm by subjecting the organism to NH_3 or CO_2 , but rather that of some of the inclusions or the vacuoles which were stained. These experiments can illustrate the "trap mechanism" and do not support BETHE's theory in toto.

M. M. BROOKS showed that the dye, 2,6-dibromo phenol indo-phenol, an acid dye, penetrates at a faster rate and reaches a higher equilibrium in the sap of *Valonia* when the sap is acid than

when it is alkaline. This agrees with BETHE's theory. Methylen blue, a basic dye, penetrates more rapidly from an alkaline solution, but at equilibrium the concentration of methylene blue in *Valonia* is the same within certain limits of acidity or alkalinity of the external solution. Here again, the sap and not the protoplasm was measured. HERTZ (1922) also using *Opalina*, found that two groups of acid dyes would stain these organisms vitally. The first group were lipid-soluble according to NIRENSTEIN's meaning (See section on Lipoid Solubility) and the second group penetrated only if a small amount of albumen had been added to the solution. (See also UMETSU 1923.) The formation of stain vacuoles in frog erythrocytes was increased by treating the cells with protein-decomposition products, and the amount of vital stain taken up after 24 hours was sufficient for analysis. (KEDROWSKI 1935.)

ROHDE placed *Paramoecium* in acetate buffers of pH 5.6 to 5.0 and found that they took up acid dyes, claiming thereby that the reaction of a living cell was changed and this enabled the cell to take up the acid dye. HERTZ (1922) claims that only when injured does *Paramoecium* take up acid dyes. BALL (1927) also found that normal *Paramoecium* stains only with basic dyes whereas the dying animals stain with acid dyes. ROHDE (1920) fed frogs boric acid or sodium carbonate, stating that the pH of the blood was changed to 4.2 and 8.7 respectively. As a result, the acid or basic dyes were taken up more readily, depending upon the acid or basic reaction of the blood. It seems, however, that such violent treatment would result in injury, so that the normal permeability was not measured. SCHULTEN (1925) did not substantiate ROHDE's results.

Since acetates penetrate rapidly, whereas phosphates do not, (See M. M. BROOKS 1923) the experiments of HERTZ (1923) who used phosphates, would not be comparable with those of ROHDE (l. c.), who used acetates. In the former case the interior becomes acid very quickly, whereas in the latter, it does not, except after a longer time. IRWIN (1926) also used acetates to influence the penetration of brilliant cresyl blue into the sap of *Nitella*.

A number of investigators have used slices of tissues and placed them in solutions of dye and noted either the disappearance of the dye from the external solution (REDFERN 1922; MANN 1924) or the appearance of the dye in the tissue (ROHDE 1917), whereas others (COLLANDER 1921; SCHAEDE 1923) have placed

the cut end of stems into dye solutions and noted the color of cells as the dye penetrated upwards via the fibro-vascular bundles.

SCHAEDE (1923) cut off root hairs of *Hydrocharis morsus-ranae* and placed them in solutions of acid and basic dyes, noting that they penetrated faster than in the case of whole plants, owing, no doubt, to the injury produced by cutting. He also found that basic dyes stain the protoplasm from an alkaline medium but not from an acid one. Chrysoidin was the only one investigated which did not injure the plant. Acid dyes did not penetrate the whole plant except acid fuchsin, which stained the granules in the protoplasm after a time without causing a cessation in the protoplasmic rotation. REDFERN (1922) found that carrot root cells absorb basic dyes more readily than acid ones. The experiments of KÜSTER (1926, 1927) and GICKLHORN (1927) are of interest and may help the interpretation of these results. These investigators injured the epidermis of *Allium cepa* by needle pricks, after which the epidermis was placed in eosin solution. It was noted that the cells in the neighborhood of those injured became stained vitally, whereas, when no injury was produced, no staining took place. Since injury produces the exosmosis of acid substances and other unknowns, (See also CHAMBERS 1927) it is conceivable that the cells in the neighborhood of those injured were affected by substances exosmosing out of the injured cells and therefore that their normal permeability was changed so that they were capable of being colored. It may also be interpreted that the dissociation of the dye in the vicinity of the injured area was changed. Experiments with *Chara* (JOST 1927) may also be quoted in this connection. It was shown that when one cell of *Chara* was injured, the adjacent cell also showed abnormal permeability.

GELLHORN (1927) also found that erythrosin stained echinoderm eggs better from an acid solution. However, RUNNSTRÖM (1928) points out that the eggs were undoubtedly injured at the pH used, viz., 3.46 to 2.81. ALBACH (1928, 1929) found that he could stain living cells of *Elodea* and *Allium cepa* protoplasm with eosin from an acid medium and not from one having an alkaline one. DOSTAL (1928) stained a number of algae like *Caulerpa*, *Codium*, *Bryopsis*, *Udotea* and *Halimeda* with various acid and basic dyes.

The older method of allowing whole plants or cells to lie in solutions for days until colored, seems to indicate that coloring

took place only when sufficient injury arose. Few organisms can withstand successfully a prolonged treatment under abnormal conditions, even if a short exposure to unusual environment is without irreversible injury

FINK and WEINFURTNER (1932) stained yeast cells with methylene blue and found that the number of cells dyed depends upon the pH of the solution. ADAMS and ROBBINS (1935) noted the change in toxicity of dyes to yeast depending upon the pH of the solutions. GENAUD (1930) showed the influence of pH on the uptake of dyes by yeast cells. YURI (1928) thinks that the affinity between bacterial cells and dyes is controlled largely by the pH of the fluid. Acid dyes combine with bacteria from acid solutions, and basic from alkaline.

PLOWE (1931) found that pH indicators do not penetrate such plant cells as onion cells, leaf cells of *Elodea* and others. After these dyes were injected into the vacuoles or the protoplast they did not diffuse into the surrounding constituents of the cell. MORITA and CHAMBERS (1929) on the other hand, were able to stain the various components of *Amoeba* with methyl red by placing the animals in solutions of this dye.

BALL (1926) states that the only dyes staining the cytoplasm of living *Paramoecium caudatum* belong to the basic group. Acid dyes did not stain.

KEDROWSKI (1934) allowed frog spawn to grow in dilute neutral red solutions and found that the tadpoles began to be colored while still within their gelatinous covering. At first granules of pigment were formed. Then basophil granules were produced by segregation of material free in the cytoplasm formed under the influence of neutral red.

The penetration of certain basic and acid dyes into red blood cells was studied by JURIŠIĆ (1927). The concentration of dye in the external solution was analyzed and it was assumed by differences from the original concentration, that the dye penetrated into the cell, although no color could be found in the cells. The author assumes that the dye color is hidden by the color of the hemoglobin rather than that it is reduced by the cells.

KINDRED (1935) studied the reactions of hemoglobiniferous cells to acid and basic dyes under varying conditions of H-ion activity.

Effect of the pH of the medium on the vital staining of vegetable cells has been studied by GUILLIERMOND and ORATON (1934) and by HUREL-PY (1934).

RAWLINS and SCHMIDT (1929) have titrated solutions of gelatine and various dyes and find that the union takes place in stoichiometric proportions.

STRUGGER (1935) concludes that BETHE's theory is not applicable to the whole cell but only to the various parts of the cell. He found that when plants were placed in neutral red, which was dissolved in distilled water more acid than pH 6.4 the cell membrane was stained, but if the dye was dissolved in a solution weakly alkaline, the sap became colored.

STEARNS and STEARNS (1931) and STEARNS (1933) have tried to interpret the staining reactions of dyes in acid or alkaline solutions on the basis of the amphoteric nature of the protoplasm and the chemical combination between dyes and substances. These authors (1930) have shown that the conductivity was decreased when dye ion and protein ion were mixed and present this as direct evidence of the ionic combination between dye ion and protein ion. They also noticed the displacement of H ions from unionized protein by dye cation or of OH ion by the dye anion. NAYLOR (1926) used imbedded sections of plant tissues and found that on changing the H-ion concentration the plant tissues responded like a protein at the isoelectric point. Acid dyes were retained in acid solutions and lost in alkaline ones, and vice versa with basic dyes.

MOMMSEN (1926) using gelatine with basic or acid dyes concluded that penetration depended upon the charge on the gelatine. This agrees with the concept of KELLER (1932), and with the work of LOEB (1915—1920) on gelatine.

PFEIFFER (1930) has attempted to obtain the isoelectric point of naked protoplasm from ripe fruits of *Solanum nigrum* by means of indicator dyes. FRENCH and WRIGHT (1931) grew cultures of *Pseudomonas tumefaciens* and *Radiobacter* at various pH values and found that the greatest absorption of congo red took place at pH 7.6, which is near the isoelectric point of the dye. PFEIFFER (l. c.) believed that the staining properties of a cell were due to a combination of the dye with tannin, as, for example, the precipitate which methylene blue produces in *Spirogyra*. However, other cells, such as *Allium*, have little or no tannin and still dyes penetrate.

VAN WISSELINGH (1914) has demonstrated intravital precipitates in *Spirogyra* by means of basic dyes, such as neutral red, methylene blue, janus green, etc. He found that the vacuole stains at first homogeneously, but that a "precipitate" gradually forms which may hold all the visible color, leaving the surrounding sap uncolored. On the addition of weak acetic it is dispersed.

SCARTH (1926) in a treatise on storage of dyes by living cells, inclines to the view that basic dyes combine with some colloidal material in the sap as well as in the cytoplasm; that the colloidal material behaves as an ampholyte, and its other physical properties suggest that it is partly hydrated lipid. It is rather difficult to visualize this mechanism for such a cell as *Valonia*, in which the organic material of the sap is practically negligible, consisting in some species of almost a pure solution of KCl. And yet this sap was able to take up the acid dyes (indophenols) and the basic dyes, methylene blue (BROOKS 1926) in considerable concentrations. In the case of acid dyes SCARTH (1926a and b) was able to produce rapid penetration of eosin by plasmolyzing the cells of *Spirogyra*. He attributes this to a change in the permeability of the plasma membrane.

BENOIST, GOLBLIN and KOPACZEWSKI (1929) have tried to establish a relationship between the penetration of acid dyes and plant cells and basic dyes and animal cells.

It is difficult to evaluate the method of plasmolysis which has been used so extensively in studying the permeability of dyes to cells. The organization of the cell seems to involve protoplasmic strands and adhesions to the wall. When the dye penetrates through the wall, in some cases at least (See SCARTH 1926) the dye seems to permeate the protoplasm, staining the nucleus, protoplasts and sap. This process is easily reversed under proper conditions and the dye exosmoses again. Plasmolysis under certain conditions does not consist in irreversible injury, but the plant seems to be able to recover and live normally afterwards.

Permeability to dyes of parts of cells. Many of the earlier papers do not differentiate the locality of staining when plant or animal cells have been stained. A color inside was observed without reference to the region colored and this fact was sufficient to indicate that the plasma membrane surrounding the cell was permeable to the dye which was being studied. In the case of oxidation-reduction indicator dyes, of course, this de-

duction was not always justified. If the reducing intensity of the tissues was sufficient to keep the dye reduced, in cases where reduction means absence of color, then without proof, one would not be able to ascertain whether the dye penetrated. Usually, however, there are some inclusions in the cell which stain, since the reduction potential of the various components of the cell are different. In that case, no matter what inclusions were stained inside the cell, the dye would of necessity have to penetrate to reach them. If, however, the wall is deeply stained, as is found in some plant cells, then it would be difficult to see whether the dye penetrated through this into the other parts of the cell, unless some differential staining occurred. If one is determining the permeability of the plasma membrane surrounding the cell alone, it is not necessary to observe the regions which are being stained. The late papers have, however, tried to differentiate the properties of the components of the cell by means of vital staining.

Plants: **PALTAUF** (1928) stained cells of *Allium cepa* with erythrosin and eosin and noted that the nuclei were colored while the sap was not. He thinks that the staining of the cytoplasm with chrysoidin, which has been often obtained by other workers, is due to injury. **WANKELL** (1921) showed that some dyes stain granules and others the cytoplasm. He also noted that some dyes become reduced by the protoplasm. **DIETRICH** (1929) used *Mucor*, placed the cells under the microscope and colored only a part. He noted the rate of diffusion of the color over a definite distance. He used methylene blue and neutral red for coloring the sap and chrysoidin and eosin for coloring the protoplasm. **STRUGGER** (1931) stained cells of *Allium cepa* with erythrosin and believes that this dye increases the permeability of the outer plasma membrane.

BECKER (1932) stained the cell plates of the pollen cells of *Tradescantia* with neutral red and methylene blue during division.

WEBER (1930) stained the vacuoles of the epidermis cells of *Vicia faba* with neutral red.

HALIK (1931) placed eggs of *Thymallus thymallus* L. and larvae of *Coregonus exiguus* in various solutions of dyes and noted the selective coloring of parts of the organisms.

SALKIND (1929) has also used neutral red and noted that the granules are stained in the case of two larvae, *Corethra* and *Chironomus*.

PRAT (1931) stained the cell walls and the crystals in cells of *Oedogonium* by methylene blue and the inclusions of *Poly-siphonia* with neutral red.

ALBACH (1929) showed the effects of the vital staining on the respiration of some dyes upon *Elodea* cells. He found that when the protoplasm was stained by chrysoidin and eosin, that the respiration was decreased. Methylene blue and neutral red color the sap and increase respiration. There seems to be some relation between the respiration and the effects of dyes which produce staining. More experiments would be of interest.

BRAUNER (1933) states that living algae may remain in solutions of methylene blue and janus green for several hours without absorbing the dye, but that dead cells absorb the color quickly. The writer (BROOKS, unpublished) has noted that in the case of *Nitella* and *Valonia*, the wall always becomes quickly colored by methylene blue in living cells. This can be washed off without apparent injury if the criterion of survival for many days following is considered adequate.

STRUGGER (1935) has analyzed the staining of the parts of a cell in the root hairs of *Trianea bogotensis* with neutral red by changing the pH. He found that at different pH values, different parts of the cell become colored, thus showing the lack of homogeneity of the various parts of the cell.

Vital staining of animal cells has also been extensively used. NASSANOV (1932) stained the macronucleus of a number of aerobic and anaerobic infusoria. MONNÉ (1935) injected about 40 different dyes into *Amoeba* in the vicinity of the nucleus and noted that all dyes penetrated the nuclear membrane, whether acid or basic, lipoid-soluble or insoluble, crystalloid or colloidal. They produced vital staining of the nucleus only when injected. On immersion, no staining occurred. These results differed from those of PLOWE (1931) who found no diffusion of injected pH indicators from one component of the protoplasm to another. Plowe, however, used plant cells, which may account for the difference in results.

MORITA and CHAMBERS (1929) have done some qualitative experiments illustrating the difference between the permeability of nuclear and cytoplasmic surfaces in *Amoeba dubia*. They injected methyl red into *Amoeba* and found subsequently by noting the change in color, that HCl penetrates the nucleus when the

cytoplasm is injected to within a short distance from it, but that there is no penetration of HCl when the animal is immersed in it.

LUCKÉ (1925) centrifuged *Arbacia* eggs and found that staining with neutral red and brilliant cresyl blue took place only in inclusions and not in cytoplasm which remained clear

RUMJANTZEW and KEDROWSKY (1926) found that in *Opalina ranarum* and *Rhizopora* a diffuse coloring of the plasm with basic dyes does not occur but that there is an accumulation in the granules and vacuoles. Acid dyes do not stain *Opalina* even in acid medium.

DANGEARD (1921) has used cresyl blue to stain algae which were grown in the dark, such as *Chlorella*, *Scenedesmus* and *Stichococcus* and found a number of granules of different sizes which were stained in the cytoplasm. LUDFORD (1930) stained certain inclusions of cells vitally, such as GOLGI bodies and acinar cells of mouse pancreas and then killed the animals and made sections.

BAILEY and ZIRKLE (1931) have made a detailed study of the pH of living vacuoles and the effects of pH in vital staining. They removed living cells of *Pinus strobus* L. and were able to keep them alive and actively streaming for more than two months. They have differentiated two types of vacuole, depending upon whether they are stained by certain basic dyes from relatively alkaline or acid solutions (Type A) or whether they are stained by dyes from basic solutions (Type B). These vacuoles are in many cases in close proximity to each other within the same cell and provide a valuable check upon generalizations concerning penetration of certain dyes. Type A vacuoles stain more rapidly and over a wider range of acid buffers than do the Type B, which rarely accumulate dye from buffers more acid than pH 5.4 to 5.8. Type A vacuoles contain tannic acid; Type B contain no tannic acid and are stained only by those basic dyes which cannot penetrate in acid solutions. The data also suggest that Type B vacuoles contain some substance or mixture of substances that produce huge errors in colorimetric determinations of the pH of vacuolar sap. These studies show that it is necessary to determine the type of vacuole which is being experimented upon before making general conclusions as to manner of penetration of dyes.

NAYLOR (1926) has shown by histological methods that tissues which were fixed, stained with either acid or basic dyes

depending upon whether the solutions used in washing after staining were acid or basic. From these experiments it would seem that at least tissues which had been killed and fixed had no basophilic or acidophilic nature, but that the method of staining was entirely responsible for the results obtained in staining.

The problem of differential staining of living and dead cells, including bacteria, yeast, spores and red blood cells, has been studied more recently by GAY and CLARK (1934) who have given a bibliography of the earlier work. See also FINK and KUHLES (1933) and DÖRING (1935) for *Allium cepa*.

Methylene Blue

Methylene blue, which has been extensively used in staining, is a highly dissociated basic dye (CLARK 1928) subject to oxidation when placed in alkaline solutions or on standing in air. These oxidation products are lower homologs (HOLMES 1927; HOLMES and SNYDER 1929), are weak electrolytes and have the ability to penetrate tissues with ease. For this reason, the older staining reactions with methylene blue were done with "aged" dye rather than with that freshly made up. Methylene blue also comes mostly in an impure state and most of the experiments have been done with such dye.

Since this dye is dissociated at all pH values, the factor of molecule vs. ion should not enter into consideration. However, this dye was found to penetrate cells of *Valonia* (See BROOKS 1926, 1927, 1929) at a faster rate from alkaline solutions at pH 8.0 than from acid ones. The difference in rate in this case may be due to effects of pH on the plasma membrane rather than on the dye, unless the dye becomes aggregated at certain H-ion concentrations. The more rapid penetration from very alkaline solutions may be due to the penetration of the lower homolog, trimethylthionine, which is formed in alkaline solutions, thereby augmenting the rate of penetration of the methylene blue and making it appear that the rate increases with increasing alkaline reaction. IRWIN's (1928) experiments showed that trimethylthionine penetrates relatively rapidly; that methylene blue from alkaline reactions (pH 9.0 to 10) penetrates rapidly and that impure dye which contains lower homologs appears to penetrate at a faster rate than pure dye. Of course, in addition, at such high pH values there is danger of injuring the cells so that permeability

increases and the dye is allowed to enter at a faster rate. IRWIN's results are attributed to these reasons.

Methylene blue is considered as not staining living protoplasm but only certain cellular inclusions or vacuolar sap. This lack of color in protoplasm may be caused by the oxidation-reduction potential of the protoplasm as a whole, whereby the dye becomes reduced to the colorless form. The fact that it passes through protoplasm and is again blue in the sap of *Valonia*, shows that the sap has a slightly higher redox potential than the protoplasm.

When tannic acid is found in the cell, according to PFEFFER (1886), a basic dye may penetrate and combine with it. PFEFFER studied the penetration of protoplasm by aniline dyes. He noted that methylene blue accumulates in granular masses in the cell sap in *Spirogyra* when placed in a solution containing one part of methylene blue in 100,000. This accumulation results from the transformation of the substances after entry into a form which cannot again pass out, thereby making room for the further entry of the pigment. This phenomenon is of the greatest significance in relation to the absorption of some materials by the plant. In the case on *Lemna*, the dye unites with tannic acid and tannate of methylene blue is incapable of escaping. If a little citric acid is added to the water after a short time the blue color in the cell disappears. A process the converse of storage takes place. The citric acid enters the cell and unites with methylene blue. Since the citrate of methylene blue is capable of penetrating protoplasm, all the blue color in the cell undergoes exosmosis. In the case of certain other cells, such as *Valonia*, there are no substances present with which dyes can combine, but penetration into sap takes place.

Then again, some dyes penetrate cells but are reduced inside and lose their color. A superficial view would be interpreted to mean that the dye had not penetrated, whereas if one added an oxidizer, such as traces of NaOH, the color, if present, would again be oxidized. Experiments of M. M. BROOKS (1926) on the dye 2,6-dibromo phenol indophenol, one of the oxidation-reduction indicators, illustrates this point. The dye penetrated and became reduced in the sap. Upon the addition to traces of NaOH, the blue color again reappeared.

Relation of dyes to other processes

Dyes have been used in various connections — vital staining from the point of view of cancer therapy, effects on respiration, which have been barely touched in this monograph, the effects on growth, etc. These are only indirectly concerned with permeability studies and have for this reason not been given here.

WELLS and SHERWOOD (1934) in a study of the selective phagistatic action of dyes for 12 bacteriophages state that there appears to be a correlation between the selective action of the dyes on bacteria and on their respective phages.

THORNBERRY (1931) has attempted to define the chemistry of the effects of bactericidal action of dyes by isolating from 12 dyes the grouping of the molecule which is found in common.

The chemistry of the various parts of the cells, including inclusions, nucleus, granules, etc., is not sufficiently known to make definite statements concerning their staining ability. It is interesting, however, to note that some dyes are able to pass from the cytoplasm into the various differential parts of the cell and produce definite coloration. In some cases the color of the dye may be too dilute to be observed in the cytoplasm and only as it becomes concentrated does it become apparent in the special structure of the cell. In other cases it may become reduced in the cytoplasm and on penetrating into the specialized part of the cell, become oxidized again and produce color. However, it seems apparent that the same explanations with reference to penetration of acid and basic dyes into cytoplasm of cells would also apply in the case of special structures, that the same laws of permeability applying to boundaries of the cells between the cell itself and the external solution, would also apply to surfaces between cytoplasm and the structures within the cells; and that the effect of acids and bases changes the properties of the dyes and also those of the limiting membranes.

Experiments such as those of ROBINSON (1935) should help greatly in solving some of the problems in connection with the permeability of dyes. He made studies of the osmotic pressure, diffusion and conductivity of highly purified solutions of benzo-purpurin 4 B, bordeaux extra, congo red, congo rubin and methylene blue. It was concluded that the micelles of benzo-purpurin 4 B contain at least 10 particles with 25% included sodium; a bordeaux extra solution showed appreciable association

in 0.5% solution; congo red has particles somewhat smaller than benzopurpurin 4B; methylene blue associates into micelles sufficiently to make this evident in the conductivity curve.

Effect of vital staining (including methylene blue) upon respiration. Any explanation of the effect upon the permeability of these substances must involve their effects upon respiration. As early as 1911 PALLADIN, HUBBENET and KORSKOW found that seedlings of *Pisum* kept for 24 to 48 hours in a solution of saccharose alone without the addition of dye. MEYERHOF (1917) found a similar increase in the respiration of yeast and *Staphylococcus*. More detailed observations were made by GENEVOIS (1928) and ALBACH (1929). GENEVOIS used some of the redox indicators and found that the respiration was increased about threefold when methylene blue was added to *Protococcacea*, but that when the dye did not penetrate, as in the case of indigo carmine, no increase in respiration was noted. He concluded that a dye must penetrate in order to influence the respiration, and that therefore the effect was not a cell-surface phenomenon.

ALBACH (1929) used a variety of dyes, basic and acid, those which stained sap and those which stained protoplasm only, and various combinations. His results are interesting.

He found that methylene blue increases the respiration of cells of *Allium cepa* about 100%. This is a basic dye and stains the sap. Fuchsin S also increases the rate of respiration about 30%. This is an acid dye and stains the protoplasm. Eosin (an acid dye staining the protoplasm) decreases the rate, while chrysoidin (a basic dye which presumably stains protoplasm) decreases the rate of respiration.

The position in the redox potential scale seems not to affect the rate of respiration, since methylene blue, which is easy to reduce, causes an increase in respiration, while chrysoidin, which is also easy to reduce, causes a decrease; whereas, neutral red, which is difficult to reduce, produces an increase.

He also found that staining is not essential at first in producing an increase in the respiration, since the increase in respiration has been observed in plants even before the dye has accumulated. He considers this contrary to the conclusion of GENEVOIS (1928). However, this may be caused by actual staining in quantities too small to be observed. In those cells which do not stain, such as indigo carmine, according to GENEVOIS (1928), no in-

crease in the respiration has been found. It is tempting to point out a relation between the staining of protoplasm and a decrease in respiration (as pointed out by ALBACH) but there are too few examples yet to make definite conclusions.

In the same way an increase in respiration by methylene blue was found in the case of mammalian erythrocytes by HARROP and BARRON (1928). This can also be correlated with the earlier experiments of HEYMANS (1923) who showed that methylene blue increased the temperature of dogs when injected intravenously.

It has since been demonstrated by M. M. BROOKS (1932 et seq.) that methylene blue acts as a catalyst for the oxidizing enzymes of the tissues.

All these facts are interpretative of the effects of dyes upon tissues and the interdependence of permeability upon metabolism.

The effects of electrolytes on the penetration of dyes

The very marked effect of electrolytes on the penetration of dyes into living cells has been extensively studied. There are at least two factors to be considered; the effect of the electrolytes upon the dye and that upon the semipermeable membrane itself. The colored portion of the dye has either a positive or a negative charge; in general, the positively charged dye ion is taken up by the cell and the negatively charged ion is not. They are transformed from one to the other by change in pH. The basic dyes are, for example, obtained as the Cl salt, but possess through free OH—COOH groups the properties of acids. Electrolytes also neutralize the charge, depending upon the concentration used. It seems reasonable, therefore, to attribute, at least in part, the effect of electrolytes to their action on the dye.

The results obtained by adding electrolytes to dyes have been recently analyzed by VALKO (1935) in diffusion experiments of dyes in water and in NaCl solutions. The effects of the electric forces were as follows: azogrenadin S was found to be almost completely dispersed into single molecules; benzopurpurin 4B, congo red and chicao blue 6B are aggregated into ionic micelles. This aggregation increases on raising the salt concentration. Experimental evidence shows that frictional resistance of large colloidal ions against osmotic forces is smaller than against electrical forces.

On concentrating the dye solution an increased aggregation results for several reasons; the law of mass action requires a displacement of the equilibrium, and the increased number of ions from the dissociating dyestuff gives rise to a salt effect.

The action of electrolytes has also been shown graphically in certain experiments with filter paper in which the ascension of dye was measured. Aside from special cases, such as that of Fe, it was found that in general electrolytes prevented dye from rising in the filter paper as compared with that rising in the controls. In some cases visible precipitation or flocculation was observed. (KOPACZEWSKI and ROSNOWSKI 1928.)

The effect of electrolytes on the penetration of basic dyes is different from that in the case of acid dyes. Most of the investigations show that electrolytes retard the uptake of basic dyes by living cells and that the effect increases with the concentration and the valency of the cations, whereas, in the case of acid dyes, electrolytes seem to increase their ability to stain cells. Since most of the acid dyes are highly dissociated, the dye anion penetrates with difficulty. The basic dyes, however, are taken up since the colored ion is a cation and is able to pass more easily through protoplasm.

The mechanism of the action of electrolytes must be related either to the neutralization of the electric charge on the colloidal particles by the opposite charge of the electrolytes on the precipitating ion, or an aggregation of some sort may occur not large enough to produce precipitation. These effects are equivalent to abolishing or reversing the charge on the dye particle, thereby increasing its ability to penetrate, in the case of the acid dyes, and reversing it in the case of basic dyes. Experimental data show that these results occur, however, whether by this mechanism or not, has not been proven.

In the following pages some examples with basic dyes and acid dyes in the presence of electrolytes are given.

Basic dyes. Szűcz (1910) used *Spirogyra* and observed that the uptake of aniline dyes is hindered by the presence of electrolytes in the external solution and concluded that the antagonistic effect depends upon the concentration and valency of the cation. ENDLER (1912) found that the uptake of methylene blue and neutral red was hindered by solutions of electrolytes more concentrated than 0.4 M. MANN (1924) used thin slices of mangold

root tissue and analyzed the external solution for concentration of dye under varying concentrations of electrolytes. He also found that electrolytes decrease the rate of uptake of methylene blue, neutral red and orange G. As the valency increases, the effect is greater. PRÁT (1927) found that the absorption of cresyl blue and methylene blue by agar was reduced by chlorides, the extent depending upon the valency of the cation.

MANN used NH_4Cl as the monovalent electrolyte. Since this has the power of penetrating rapidly into the interior of the cell, thereby changing the pH inside, it would have been more interesting if he had used a type of electrolyte such as NaCl which is known not to change the interior pH of the cell.

MANN, like SZÜCZ, concluded that the magnitude of the antagonistic action is a function of the valency of the salt and its concentration. HÖBER and MEMMESHEIMER (1923) and TANAKA (1924) showed that less rhodamin B or methyl violet or methylene blue (basic dyes) are taken up by red blood cells if washed with an isotonic solution of glucose or glycocoll, than with a solution of NaCl . TANAKA (1924) further showed that the opposite is true for acid dyes, especially for lipid-soluble ones.

MACARTHUR (1921) finds that neutral salts retard or actually prevent staining of all parts of planarians with methylene blue and other basic dyes. CaCl_2 is more effective than NaCl . His theory is as follows: Previous absorption of acid or neutral salts tends to discharge and aggregate electronegative colloids and accordingly, both in vivo and in vitro there is a decrease in basic staining and an increase in acid staining, while inversely, alkaline solutions favor basic staining and diminish acid staining.

SCARTH (1923) states that methylene blue fails to stain the cell wall of *Spirogyra* in the presence of trivalent salts. BORNSTEIN and RÜTER (1925) found that neutral salts (NaCl , LiCl , RbCl , MgCl_2 and CaCl_2) caused discoloration, particularly in the vacuole of *Paramecium*. The salts of the heavy metals in concentrations of 1×10^{-5} to 1×10^{-7} were without effect on the vital staining.

IRWIN (1926) found that brilliant cresyl blue penetrates less readily into the sap of *Valonia* from solutions containing salts. The effect of electrolytes on the excretion of certain dyes such as neutral red, methylene blue and brilliant cresyl blue by protozoa was studied by ANDREYEVA (1929). He noted the time at

which the first drop of dye was being excreted. A lyotropic series was obtained which agrees with that of HAMBURGER (1890) on red blood cells and LILLIE (1909) on the action of salts on the excretion of the pigment of *Arbacia* eggs.

ENDLER (1912a) also studied the influence of neutral salts on the uptake of dyes. He found that electrolytes such as NaCl KCl and others increased the penetration of methylene blue and neutral red into living cells with an optimum penetration when the electrolyte was between 0.3 and 0.4 M. Below and above this concentration there was a diminution in the concentration of the dye found in the cell after a time. Since there are no time curves, it is not possible to conclude whether the rate of penetration was affected or the final equilibrium alone. Also, since the plants were allowed to remain in these solutions at least 12 hours before observations were made, it seems when such fresh water plants as *Elodea* and *Vallisneria* were placed in the higher concentrations of salt solutions, especially those above 0.01 M, that there must have been considerable injury so that the increased concentration of dye observed in these plants would have been due to an abnormal permeability. He concluded that the effects of the cations are not so different from each other except that Al hinders the uptake of dye more strongly. This agrees with the results of SEKI (1934). SEKI, believing the charge on the membrane to be responsible for staining reactions, found that in neutral and alkaline reactions several mordant dyes, whose isoelectric points were in the acid range, were adsorbed either weakly or not at all by collodion or SiO_2 which are similarly charged. These dyes stain tissues histologically in the usual manner. If the charge is reversed by the addition of Al or Cu salts, then the tissues are stained by basic dyes.

BROOKS, M. M. (1927) made direct determinations of the penetration of a triphenylmethane dye, dahlia, through the protoplasm into the sap of a unicellular plant, *Nitella*. The plants are of such size that the sap can be extracted without complications and the concentration of dye determined colorimetrically. It was found that the chlorides of Na, K, Mg and Ca have a retarding effect in concentrations from .05 to .0125 M upon the penetration of this dye. The bivalent cations are more effective than the univalent. A slight degree of antagonism was obtained between CaCl_2 and NaCl, but not sufficient to permit the dye to

penetrate at a normal rate. No pH buffers were used to complicate the results. Higher concentrations produce injury. More recently OETTINGER and PICKETT (1932) showed that PO_4 buffers in concentrations of .008—.048% produce a marked reduction of ionized Ca. This can be lessened by addition of bicarbonate buffers.

GELLHORN (1931a and b) has also noted the antagonist effect of electrolytes on the penetration of several acid and basic dyes into *Strongylocentrotus purpuratus*.

It is of interest to note the experiments of PORT (1926) in this connection. He observed the color changes in petals of *Viola tricolor* when NH_4OH and electrolytes were placed in the external solution. Under these conditions alkali salts with univalent cations hinder the penetration of OH ions; those with bivalent cations are still more effective. These results are in close agreement with those of many basic dyes which are weak electrolytes.

The very marked effect of electrolytes in altering the charge on surfaces has been studied by BETHE (1919). He has determined for a number of basic and acid dyes the influence of electrolytes upon the charge of the dye. He found that in general the addition of alkali or of neutral salts to the aqueous solution of methylene blue and other basic dyes caused a partial movement to the anode. The bivalent sulphate ion in smaller concentrations produces the partial anodic drift of methylene blue more than the univalent chloride ion. FÜRTH (1925) used dyes in a cataphoretic apparatus and found that methylene blue migrates to the anode in alkaline solutions and to the cathode in acid solutions.

SÜLLMANN (1931) determined the solubility of methylene blue in equinormal salt solutions of similar cations and different anions and found a salting-out effect following the familiar lyotropic series, similar to those found in the experiments of KAHN (1926) and is not dependent upon the valency. He has shown that the presence of electrolytes has a strong influence on the solubility of methylene blue. He used .025% methylene blue in 4 N concentration of NaCNS, NaBr, NaNO_3 , NaCl, NaAcetate, Na_2SO_4 and NaF, thereby changing only the anion and keeping the cation the same. He believes that there is a similarity between cataphoretic experiments with living cells and tissues and the direction of transport in the cataphoretic apparatus; and that adsorption of the H or OH takes place on the dye molecule, presumably as charges surrounding the molecule.

Acid Dyes. An example of the effects of electrolytes on acid dyes is the work of ALBACH (1928). He was able to stain the cells of *Allium cepa* with eosin to a greater extent when electrolytes were present. The higher the valency of the cation the greater was the uptake. This effect is opposite to that found with basic dyes. There is also an effect of pH here. The lower the pH, the more dye is taken up. This effect is as expected, if the dissociation of the dye is repressed and a greater concentration of molecules is available. Above pH 7.2 no staining occurs unless one adds KNO_3 , acetic acid or AlCl_3 , which presumably have additional effects on the protoplasm.

SCARTH (1926) also finds that NaCl and CaCl_2 are effective in causing increased rate of penetration of eosin in concentrations greater than 0.1 M, whereas in concentrations less than this the reverse is true.

The effect of varying amounts of electrolytes on the diffusion of certain dyestuffs have shown that small amounts of added salts increase the rate of diffusion in water, whereas at greater salt concentration decreases it (SCHRAMEK and GOTTE 1931).

NEAL and PATEL (1934) have made a careful quantitative study of the effect of electrolytes with special attention to the attainment of an equilibrium absorption and to the measurement of the rate at which it is used. The greater the concentration of added electrolyte the greater is the absorption at equilibrium. When the electrolyte concentration is low, the absorption increases with increase in valency of the cation. These experiments were done in boiling vats and are not comparable to conditions found in living cells but are of comparative value and interest.

HARTLEY and ROBINSON (1931) find that the addition of electrolytes steadily decreases the rate of diffusion of dyestuffs in water and point out that the relation between particle size and the rate of diffusion is by no means a simple one, since the dyestuffs must be regarded as colloidal electrolytes and not as simple colloids.

An interesting relation in this connection concerning the free diffusion of an acid dye in water has been found.

When an aqueous solution of the Na salt of p-sulfo-benzeneazo-benzeneazo-6-benzoylamino-1-naphthol-3-sulfonic acid and NaCl is allowed to stand 1 month at 25°C . the rate of diffusion of the color ion is markedly affected by the presence of NaCl in the solution and an increase of 25 to 50% in the diffusion constant

occurs (LENHER and SMITH 1934). This relation is interesting in comparing the effects of electrolytes on the uptake of acid dyes by cells. It would be interesting to know whether other sulfonic acid dyes reported in the literature as not penetrating living tissues could be taken up if an electrolyte, as for example, NaCl were present. In the experiments of LENHER and SMITH the dye had absorbed a large portion of the electrolyte originally present. Whether the charge had been neutralized, thereby changing the dye to the molecular disperse state, is not stated. However, such a change would account for a more rapid penetration of acid dyes in the presence of electrolytes in living tissue.

Since some of the acid dyes which do not penetrate are highly dissociated sulfonated compounds, the adsorption of an electrolyte such as NaCl and a subsequent neutralization of the charge on the dye might account for its subsequent penetration.

It might not be out of place to suggest at this time that this explanation might also account for the ability of the dye phenol red, which is an acid sulfonated dye, to be concentrated in the proximal convoluted tubules of the kidneys because of the presence of NaCl which is a product of importance in excretion by the kidney, and its change in state to that of one either more molecular- or more ionic-dispersed.

On the other hand, KAHO (1926), using no dyes, showed the action of the alkali salts upon the coagulation of protoplasm. He determined the time and concentration of solutions required to kill the cell, noting as criterion for living, the ability to be plasmolyzed. The order of killing with salts of different cations was: $Mg > Ba > Sr > Ca$, and those with different anions: $CNS > Br > NO_3 > C_2H_3O_2 > Cl > SO_4$.

Interpretation. It seems from the above data that the presence of electrolytes in experiments with dyes affect not only the dyes themselves but also the protoplasm, and both factors should be considered in an interpretation of their effects.

The addition of electrolytes to solutions of dye changes the aggregation of the colloid in different ways depending upon the chemical configuration of the dye. For example, LENHER and SMITH (1934) find that the diffusion of an aqueous solution of the Na salt of p-sulfo-benzeneazo-benzeneazo-6-benzoylamino-1-naphthol-3-sulfonic acid is increased by the presence of NaCl in the solution into which diffusion occurs; that this is marked in aged

solutions. They state that this indicates that the dye has adsorbed a large portion of the electrolyte added.

VALKO (1935) has studied the diffusion of orange II, azogrenadin S, benzopurpurin 4B, congo red and chicago blue and found that aggregation is always greater in the presence of a salt solution. The first two dyes were found to be completely or almost completely dispersed into single molecules; the remaining dyes were aggregated to ionic micelles. This aggregation increases on raising the salt concentration.

ROBINSON (1935) used meta benzopurpurin, benzopurpurin 4B, bordeaux extra and congo red. On increasing the concentration of NaCl he found that bordeaux extra only shows a small degree of anion aggregation. He describes the structure of the dyes as consisting of a micelle, with ions having a definite orientation as in a micro crystal. The dyes associate with increased dye concentration. In the case of methylene blue, the association begins at about a concentration of .0004 M according to the conductivity curves and increases as the concentration increases. A solution of such a dye as methylene blue may therefore be considered only a "true solution" in concentrations below .0004 M (Fig. 13).

In conclusion, the addition of electrolytes, changes in concentrations of dye used, and the purity of the dye are important considerations in the study of their penetration into cells. Most of the impurities are presumably salts, so that their presence would be comparable to the addition of electrolytes. The valency of the electrolytes is also important, the effect increasing with increase in valency. There is a general "feeling" that electrolytes increase the uptake of acid dyes and decrease the uptake of basic dyes. A few recent experiments on salt effects upon dyes alone are added.

Penetration of the Sulfonate Radical. It has been pointed out by various investigators that the sulfonate radical **does not penetrate living cells**. The high degree of dissociation of the dye is evidently not the cause of this lack of penetration, for the reason that there are other dyes highly dissociated which do penetrate. One must, therefore, conclude that the chemical or physical nature of the radical must be responsible. They are for the most part difficultly soluble and highly colloidal.

OVERTON (1899) was perhaps the first to note that sulfonic acid does not penetrate living cells. NIRENSTEIN (1920) states

that these dyes penetrate into the living cell of *Paramoecium*. HÖBER (1909) found Echtrot A and Tuchrot 3 GA penetrated the intestinal epithelium of frog and red blood cells of beef. HERTZ (1922) states that Tuchscharlach G is taken up by the intestinal parasite of frog, *Opalina ranarum*. RUHLAND (1912, 1913, 1914) states that sulfonic acid dyes are as permeable for protoplasts as basic dyestuffs. This is contradicted, however, by COLLANDER (1921), who found that they do not penetrate plant cells readily, and attributes the results of RUHLAND to the state of cells used.

The NEEDHAMS (1926) called attention to the fact that the redox indicators having the sulfonate radical did not penetrate certain protozoa. M. M. BROOKS (1926, 1931) noted that the indigo sulfonates and 1-naphthol-2-sulfonate-phenol-indophenol did not penetrate *Valonia*. HARVEY (1929) showed that those dyes having a sulfonate radical did not produce dimming of luminescent bacteria until all the O_2 was used up, whereas the other indicators caused a gradual dimming. BARRON and HOFFMAN (1930) found that indigo sulfonate did not penetrate starfish eggs, and, therefore, did not affect their O_2 consumption.

CHAMBERS, COHEN and POLLACK (1931), and CHAMBERS, BECK and GREEN (1933) also suggested that the sulfonate radical was responsible for the lack of penetration of sulfonate dyes into echinoderm eggs in immersion experiments.

CHAMBERS and KERR (1932) found that varying the pH of the external medium had no effect on the penetration of the sulfonphthalein indicators in the case of *Limnobium* root hairs.

There is unanimity of agreement¹⁾ that the sulfonate radical does not penetrate. In this connection it may be of interest to call attention to the 2 redox indicators of MICHAELIS and EAGLE (1930); gallophenine, which is a sulfonated oxazine, and brilliant alizarin blue, which is a sulfonated thiazine. These redox indicators are in the intermediate region of the redox scale near methylene blue. Since they contain the sulfonate radical they are valuable in those experiments in which it is desirable to poise the outside solution at a suitable region without affecting the processes of the cell itself.

¹⁾ Exceptions to this rule seem to be the proximal tubules of the chick and other animals which allow the sulfonated dyes to pass through.

HECHT (1935) has studied the elimination through various organs of certain dyes of the sulfo-groups. He showed certain irregularities in the distribution of drugs and other substances in the organism. If there are 1 to 3 sulfo-groups present in the dye, elimination is through the liver if coarsely dispersed, and through the kidneys and liver if finely dispersed. If there are four or more groups, there is permanent storage in the liver if coarsely dispersed, and elimination through the kidneys if finely dispersed. Mono- and di azo dyes acted alike and the position of the sulfo-groups did not seem to exert any influence. He thinks that the influence of the number of sulfo-groups is probably due to the hydrophilic property of the ionized sulfo-group.

Permeability of the Kidneys to Dyes. Dyes have been extensively used in the study of kidney structure to differentiate functional regions and their selective permeability. To give an adequate discussion of the literature of the permeability of kidneys to substances would require more space than has been allotted. Therefore, only a very brief summary and a few of the more recent references will be given of the problems which have been connected with the study of dyes in relation to the permeability of various regions of the kidney. In addition to the factor of permeability, that of secretion must also be considered. A dye may pass through a membrane successfully, only to be trapped, concentrated, and retained in the renal cells or passed back into the blood stream. The process of concentration may be due in part to the re-absorption of water from the cell containing the dye, or to the accumulation of dye inside the cell. The term "secretion" has been extensively used in this connection in the literature. It seems, however, to the writers that "secretion" should be restricted in its meaning to the actual manufacture of a chemical substance in the cell, rather than to the outward passage of a foreign substance like a dye, which has not been chemically altered by the cell, but remains in the same state as it was when penetrating.

The "secretion" of dyes by certain granules, as shown in the salivary glands of *Chironomus* larvae (GOLGI apparatus) (PARAT and PAINLEVÉ 1924; BEAMS and GOLDSMITH 1930; and KRJUKOWA 1929) is an interesting phenomenon. These bodies are at first small and then swell and burst out their contents. An interesting analogy in plant cells has been reported by WEIER (1933) in

which he describes how certain granules in the protonema of *Polytrichum commune* located in the cytoplasm, accumulate neutral red, swell, fuse and then become incorporated in the sap vacuole. When very dilute solutions are used, the granules accumulate stain, remain red for some time and then become decolorized, no doubt by the process of reduction.

It has been found by means of dyes, especially phenol red, that certain regions of the kidneys are differentiated as to function; for example, the proximal convoluted tubule (2nd Abschnitt) has been found to be the seat of concentration of certain dyes.

The process of "secretion" of phenol red by the kidney involves the storage or concentration of the dye in the renal cells. Phenol red does not penetrate red blood cells, but both urea and phenol red occur in the frog's renal cells in a much higher concentration than in the blood or other tissues. Both of these substances are "secreted" by the tubules of the frog kidney, but only phenol red is secreted by the mammalian kidney, urea being eliminated entirely by filtration.

MARSHALL and CRANE (1924) have undertaken experiments on the elimination of phenol red and urea by the amphibian and mammalian kidney. Phenol red is concentrated and stored in the cells of both amphibian and mammalian kidney. They conclude that these substances are secreted mainly, if not entirely, by the proximal convoluted tubules.

In the case of aglomerular kidneys, the secretion of phenol red is neither proportional to blood concentration nor dependent upon urine flow. (BIETER 1933; MARSHALL and GRAFFLIN 1932, for the goosefish; and by MARSHALL 1930, for the toadfish; MICHALOWSKI 1935; and SHANNON 1935, for dogs).

WEARN and RICHARDS (1924) and RICHARDS and WALKER (1930) were able to recover from the glomerulus of living kidney injected dyes by puncturing with a very fine capillary and extracting the dye from that location.

MOSONYI and VOITH (1934) dispute the conclusions of WEARN and RICHARDS that chloride secretion takes place in the glomeruli because the chloride content of glomerular fluid is higher than that of the plasma and believe that it is due to the absence of protein in the filtrate.

SHEEHAN and SOUTHWORTH (1934) have made a quantitative study of the elimination of phenol red by the kidney and

state that the results cannot be interpreted on the theory that the glomerules filters like a dialyzing membrane.

EDWARDS and MARSHALL (1924) observed the location of the dye, phenol red, which had filtered through the kidneys and believed that the dye was secreted and not resorbed.

LIANG (1929) studied the penetration of sulfonic acid dyes. He found that secretion is only possible when lipid dyes are allowed to penetrate the 2nd "Abschnitt" through the V. portae renalis. Lipoid-insoluble dyes can enter only through the glomeruli. Lipoid-insoluble or slightly soluble compounds can penetrate the wall of uriniferous tubules if they have a small molecular structure or ionic volume, but do not become concentrated. Anions are not able to penetrate in the same direction, resembling the action of well-known selective cation-permeable membranes. Lipoid-insoluble sulfonic acid dyes can only penetrate from the artery into the uriniferous tubules and become only slightly concentrated there, while lipid-soluble dyes can also penetrate through the walls of the uriniferous tubules and become considerably concentrated there.

HÖBER (1924), MIAMURA (1924) and YOSHIDA (1924) found that a number of substances, including acid dyes like cyanol and phenol red are not "resorbed back" into the blood stream but remain and become concentrated.

PLATTNER (1924) investigated the effect of adding a small amount of protein to a solution of indigo-carmin dissolved in RINGER solution. In the absence of protein, it is not capable of coloring the gall bladder and bile capillaries, when the bile ducts are ligatured and the liver is perfused; but when a small amount of protein is present in the external solution containing the dye, the bile capillaries stain but not the gall bladder.

The significance of this is that this dye which is an acid dye, becomes concentrated in the uriniferous tubules. This process is hindered by narcosis; while basic dyes which do not become concentrated in the tubules are not affected by narcotics. This observation is used as the basis for theorizing as to the mechanism of concentration of the dye, i. e., whether by resorption of water, as in the case of the kidneys, as stated by HÖBER (1924). DE HAAN (1922) thinks that the reason for the storage of acid dyes in the tubules of the kidneys is because some albumen-like substance in the serum penetrates with the dye and it is again

resorbed, while the dye is not. (See page 226 — ultrafilter theory).

SCHEMINSKY (1929) showed that a number of sulfonphthalein dyes and neutral red enter the tubules from the blood stream and become concentrated. Neutral red enters easily from the vein and a little less easily from the artery. Cyanol enters easily from the artery and never from the vein. He concludes that the entrance of sulfonphthalein takes place in the "2nd Abschnitt" of the uriniferous tubules. HÖBER and MEIROWSKY (1932) conclude that the concentration of dyes by the second segment of the kidney tubule is due partly to reabsorption of water but primarily to active secretion (meaning accumulation).

ROBBINS and WILHELM (1933) made a study of the mechanism of the excretion of lipoid-insoluble acid dyes by the frog kidney and found that there is a definite relation between the chemical nature of the compound and the mode of its elimination by the kidney.

HÖBER (1935) found a differential permeability in the kidney for diffusible and colloidal dyes. The excretion of water blue, a colloidal, acid dye of the triphenylmethane series was followed quantitatively in dogs by HEMINGWAY, SCOTT and WRIGHT (1935) who showed that elimination of the dye follows an exponential curve.

ROBOZ (1932) found that fuchsin S (acid) decreases the elimination of NaCl but water blue increases the elimination. He believes that the antidiuretic action is due to diminished diffusion resulting from injury to the glomerulus. NEMETH (1933) found that blockade of the tubules with water blue increased the time required for the appearance of azofuchsin and of cyanol in the urine. ROHDE (1920) in his experiments with feeding of boric acid into the blood stream found that the excretion of acid by this method was greatly increased, while that of basic dyes was greatly hindered. POHLE (1924) also noted that the excretion of acid dyestuffs through the kidneys of warm-blooded animals as dogs, is increased if the urine has been made acid by feeding acid at the same time to the animal or by intravenous injection. It is also decreased if the reaction of the urine is made alkaline by giving alkali. The excretion of alkali dyes is influenced in the same way, an increase by feeding alkali and a decrease by feeding acid. Highly colloidal dyes which are ordinarily not resorbed are resorbed and excreted under the influence of acids.

It seems from the work of CHAMBERS (1935) with chick mesonephros in tissue cultures that there are two ways in which the cells of the proximal tubules may take up dyes. One appears to be a passive infiltration of a lipid-soluble dye and the other which depends upon the metabolic activity of the cells. He found that a low temperature (3° to 6° C.) prevents the passage of the sulfonphthaleins into the tubules and suppresses the coloration of the cells by these acid dyes. On the other hand, this low temperature had no effect upon basic dyes.

What effect does a low temperature have upon the state of the electrolytes, NaCl (See LENHER and SMITH 1934, on the effects of NaCl on sulfonic acid dyes) so that they in turn would affect the state of the dye? Would it decrease their "activity"?

OLIVER and LUND (1933) have tried to determine what the effects of LOCKE's solution containing a high ratio of KCl/CaCl₂ would have on elimination of neutral red by the perfused kidneys. They found that no more dye is liberated from the cells by the LOCKE's solution containing a high ratio of KCl/CaCl₂ than is freed by that containing the usual ratio.

Since neutral red is a basic dye, it would be affected by the presence of electrolytes (See section on electrolytes) and the concentration of the dye attained in the cell would be less than that in the presence of water alone. LOCKE's solution is a balanced electrolyte solution and although a change in the ratio of KCl/CaCl₂ is effective in other connections, nevertheless, the presence of electrolytes is still the factor of prime importance. See the experiments of M. M. BROOKS (1927) in which a change in ratio of NaCl and CaCl₂ on the penetration of a basic dye into *Nitella*, had only a slight effect on its uptake by the cell.

It would have been interesting in the experiments of OLIVER and LUND (1933) if they had also used an acid dye in connection with their work and then noted the effects of the electrolytes on its penetration or liberation, since electrolytes in general seem to favor the penetration of sulfonic acid dyes (See section on electrolytes).

KELLER (1934) attributes the ability of the renal cortex and renal medulla to take up from the blood acid dyes because of their negative charge.

CHAMBERS (1935) and his colleagues have studied the relation existing between the permeability of the kidneys to dyes and the

enzymes controlling the processes of respiration and glucolysis in proximal tubules of chick mesonephros by the tissue culture method. They have used reagents which are known to inhibit the respective metabolic processes. The effect of Na iodoacetate on dye penetration in chick kidney was studied by BECK and CHAMBERS (1935). They found that the accumulation of phenol red, chlorophenol red and anisole red was retarded by Na iodoacetate, while sodium lactate, pyruvate and succinate counteracted the effect. CHAMBERS, BECK and BELKIN (1935) found that CN, H₂S and N inhibit the accumulation of phenol red in the chick kidney.

The liver is also capable of concentrating dyes. HÖBER (1932) has found that the addition of a lipid-insoluble surface-inactive organic compound to the perfusion fluid reduces the degree of concentration of dyes by the frog liver. He attributes this to an effect on the permeability of hepatic cells. (See also HECHT 1935). TADA (1933) also states that the removal of dyes from the blood is a function of the parenchymatous cells of the liver and that dyes are "secreted" by these cells into the bile.

Further details may be found in HÖBER (1926) and MARSHALL (1934).

Secretion by Gills. In perfusion experiments with the heart-gill preparation of the eel, *Anguilla vulgaris*, KEYES (1931, 1933), BATEMEN and KEYES (1932) and KEYES and WILLMER (1932) found that a concentrated chloride solution is secreted by the gills in opposition to a large concentration gradient. It was also found that changes in water content of the eel during adaptation to changed salinity are not paralleled by changes in water or osmotic concentration of the blood. In the adaptation from sea water to fresh water, and the osmotic regulation in fresh water after acclimatization, the kidney is the responsible active organ. In sea water, however, and in the adaptation from fresh water to sea water, sea water is ingested and absorbed from the gut, and the excess salt is secreted out from the gills which bear the burden of osmotic regulation.

In terms of grams of tissue, the work done by the gills of the eel in maintaining the normal blood concentration in an external environment of sea water, is of a similar order to that done by the human kidney in its function of regulating the blood concentration. The osmotic secretory activity of the gills is largely con-

fined to the secretion of chloride and a monovalent cation, probably sodium, together with a small amount of water.

Influence of Narcotics, Alkaloids, Respiratory Depressants and X-rays on Permeability to Dyes. When narcotics are present there is a general agreement in the experiments that decrease in permeability to dyes is produced, due presumably to their surface effect. The staining of petals of hyacinth by cyanol and orange G is decreased by 2% ether or 0.5—1% chloralhydrate as shown by COLLANDER (1921). *Opalina* lose their ability to be stained with dyes when 0.03 to 0.05 M isobutyl urethane is used (HERTZ 1922). See KONO (1930, review of the effects of poisons on vital staining). He also showed qualitatively that there is a decrease in vital staining with basic dyes when alkaloids are used. BORNSTEIN and RUTER (1925) found a decoloration of the dye penetrating into *Paramoecium* and other protozoa when such alkaloids as caffein, theophyllin, pilocarpin, chinine, strychnine, atropine or novocaine were present. There is no indication in this work as to whether the decoloration indicates reduction of the dye or a lower concentration. Since some of these substances are strongly dissociated and others weakly dissociated, there appears to be no direct relation between the degree of dissociation of these alkaloids and their staining ability.

CHAMBERS, BECK and GREEN (1933) have studied the effects of ether, phenyl urethane and ethyl alcohol on the anaerobic reducing power of intact eggs with cresyl blue, methylene blue and ethyl capri blue. They found that ether and phenyl urethane have no appreciable effect on the reduction but that ethyl alcohol exerts a definite accelerating influence.

They also found that KCN in concentrations which are known to be more than sufficient to inhibit oxygen consumption, has no appreciable influence on the rate of dye reductions by the eggs. When concentrated solutions such as N/10 KCN are used, there is an increase, however, in the time of reduction.

Although these experiments were based entirely upon the condition of the dye in the external solution, it has been found in previous publications that most of them penetrate into the egg.

Ross (1934, 1938), using methylene blue and KCN in experiments with *Nitella*, shows that there is essentially no effect on the equilibrium concentration of methylene blue which has

penetrated into the sap in certain concentrations of KCN and methylene blue in the external solution.

BROOKS, M. M. (1938) found that in the case of *Valonia* there is no effect on either the rate of penetration of the dye or on its concentration in the sap at equilibrium in the presence of KCN with methylene blue or 2, 6-dibromo phenol indophenol. With the former dye the pH of the external solution was that of sea water, whereas with the latter the pH of the external solution was changed to 6.0 by means of phosphate buffers.

In the case of frog kidney, both KCN and CH_2ICOOH inhibit reversibly the phenol red excretion (HÖBER and FERRARI 1933).

KOOHES, K. (1928), states that x-rays increase the permeability of the cell membrane to dyes. This may of course be due to injury of the radiation.

CHAPTER XIII

THE PERMEABILITY OF LIVING CELLS TO OXIDATION-REDUCTION DYES

Orientation

Among the dyes which promise considerable aid in helping to solve some of the problems in permeability are the oxidation-reduction indicators (CLARK 1925, 1928). Their property of becoming readily reduced to a colorless form or oxidized back to their original color gives them a special place in the study of dye penetration.

Although the more common dyes like indigo carmine and methylene blue have been used in reduction experiments for a considerable time (EHRlich 1885) it is only since the physical measurements by CLARK and co-workers (1923, 1925) of a series of these dyes that there has been an indication of the meaning of the results obtained when applied to biological material. In a classic study of a series of reversible indicators, CLARK and co-workers determined the relations between electrode potential, pH and proportion of oxidized and reduced dye of a number of electromotively active dyes and arranged them on a comparative scale in the order of their ease of reduction. By means of these indicators one is enabled to determine in physical terms the oxidizing or reducing ability of the experimental material.

There has been some criticism of the use of the term, oxidation-reduction potential, as applied to reducing or oxidizing intensity of living material. Since living material is presumably never in a state of equilibrium but constantly changing, the point has been raised as to whether the measurements published in the literature which has since accumulated, can be truly considered in the light of the thermodynamic equilibrium. The same objections could be raised in applying the term pH to protoplasm. Since protoplasm does not consist of a homogeneous system, strictly speaking, one could not, therefore, accurately refer to its pH. However, since the separate components of protoplasm

are not known; the value used refers to the resultant of the separate pH values which the separate components may have. And since the protoplasm and its separate components are in a constant state of flux or change, strictly speaking it would not be possible to assign any general value to a condition at one instant. In the same way we may consider the objections raised concerning the use of thermodynamic terms to a system not in equilibrium, such as protoplasm. On the other hand, successive measurements at different times are sufficiently reproducible by more than one method, within certain limits, so that there is some justification for the use of these terms if we do not forget the limitations imposed upon their interpretation. With this reservation in mind there seems no reason why such a term cannot be continued in order to avoid the complexity of expression and the confusion which will result from a multiplicity of terms. The symbol rH is a convenient term for setting a definite region of oxidation-reduction intensity without the necessity of specifying pH. This has been used by a number of investigators, while others have preferred to use values for E_o' at a given pH (usually 7.0) as the reference values.

Besides the original indicators on CLARK's oxidation-reduction scale, which included indophenols, thiazines and indigo sulfonates, the following dyes have been measured: phenosafranine, toluidine blue, cresyl blue, neutral red and Nile blue by VELLINGER (1929); gallocyanine, gallophenine and brilliant alizarin blue by MICHAELIS and EAGLE (1930); rosinduline by MICHAELIS (1931); several of the oxazines, salts of naphthophenazooxonium and azines by RAPKINE, STRUYK and WURMSER (1929); certain indophenols by HALL, PREISLER and COHEN (1929) and GIBBS, HALL and CLARK (1929); certain oxazines by LETORT (1932); and the viologens by MICHAELIS and HILL (1933). A summary of these indicators is included in Table XXXIV.

If we adhere strictly to the interpretation of permeability by considering only studies on penetration, then the number of references which deal with penetration of oxidation-reduction indicators is very limited. However, experiments which throw light on the state of the interior of the cell, are valuable in an interpretation of the penetrability of substances. Since the ramifications which the applications of these indicators to biological literature have produced are many and have been ably treated

Table XXXIV. Characteristics of certain dyes useful as indicators.
Taken from COHEN, B. Cold Spring Harbor Monograph I, 195—204, 1933.

Indophenols

Name	E_0 at pH 7.0	E_0	pK_0	pK_R	pK_2	Reference
Phenol-m-sulfonate indo-2, 6-dibromophenol . .	0.273	0.6906	7.40	7.12	8.93	W. M. CLARK and Co-workers 1928 do. do. do. do. do. do.
m-Chlorophenol indo-2, 6-dichlorophenol	0.254	0.6919	6.16	6.89	9.21	
Phenol-o-sulfonate indo-2, 6-dibromophenol . .	0.242	0.6834	6.07	7.01	10.22	
o-Chlorophenol-indophenol	0.233	0.6627	7.00	8.44	10.30	
2, 6-Dichlorophenol-indophenol	0.217	0.6684	5.70	7.00	10.13	
2, 6-Dichlorophenol-indo-o-cresol	0.181	0.6394	5.50	7.10	10.43	
1-Naphthol-2-sulfonate indo-2, 6-dichlorophenol .	0.119	0.5630	6.14	7.45	9.32	

Amino indophenols

System	E_0' at pH 7.0	E_0	pK_0	pK_{obl}	pK_R	pK_2	Reference
Phenol blue	0.225	0.677	4.85	high	9.88	5.96	COHEN and PHIL- IPS 1929 do.
m-Toluyene diamine-indophenol . . . $pK_3 = 2.72$; $pK_w = 13.73$.	0.125	0.567	8.07	2.31	10.32	4.96	

Indamines

System	E' at pH 7.0	E ₀	pK ₀₁	pK ₀₂	pK ₂	pK ₃	pK ₄	Reference
Bindschedler's Green . . .	0.224	0.680	11.0	3.27	6.46	5.10	—	PHILLIPS-CLARK & COHEN 1927
Toluylene blue	0.115	0.601	10.48	3.80	6.56	4.40	2.14	do.

Thiazines

Lauth's violet	0.062	0.563	11.0	low	5.30	5.85	—	W. M. CLARK and Co-workers 1928
Methylene blue	0.011	0.532	high	low	4.38	4.52	—	do.

Oxazines

Cresyl blue	+047	0.583	10.7	low	6.3	4.6	—	COHEN & PREISLER 1931; VELLINGER 1929
Methyl Capri blue	—061	0.477	high	low	6.10	4.85	—	do.
Ethyl Capri blue	—072	0.540	high	low	7.14	6.70	—	do.
Nile blue-HSO ₄	—122	0.406	9.7	low	6.90	3.92	—	do.

Indigo sulfonates

System	E' at pH 7.0	E ₀	pK ₁	Reference
Indigo tetrasulfonate	-.046	0.365	6.9	SULLIVAN, COHEN & CLARK 1923
Indigo trisulfonate	-.081	0.332	7.1	do.
Indigo disulfonate	-.125	0.291	7.3	do.
Indigo monosulfonate ¹⁾	-.159	0.262	7.8	do.

Safranines

System	E' at pH 7.0	E ₀	pK ₂	pK ₃	Reference
Pheno-safranine (Rowe 840)	-.252	0.280	4.95	5.8	STIEHLER, CHEN and CLARK 1933
Tetraethyl- " (Rowe 847)	-.254	0.355	6.4	7.7	do.
Dimethyl- " (Rowe 842)	-.260	0.286	4.9	6.3	do.
Tetramethyl- "	-.273	0.288	5.3	6.5	do.
Safranine T (Rowe 841)	-.289	0.235	4.7	5.7	do.

Rosinduline & Rosindone

Rosinduline scarlet (Rowe 827)	-.296	0.047	4.5	?	STIEHLER 1933
Sulfonated rosindone	-.380	0.25	7.5	9.5	do.

¹⁾ The monosulfate is rather poorly soluble when salts are present.

Supplement

System		Reference
Galloyanine	—	MICHAELIS & EAGLE 1930
Gallophenine	—	do.
Brilliant alizarin blue	—	do.
Rosinduline 2G	—0.161 at pH 5.0	MICHAELIS 1931
Rosinduline 2G	—0.480 at pH 11.0	do.
Toluidine blue, Azur I.	—	RAPKINE, STRUYK & WURMSER (1929)
Neutral red	—	CLARK & PERKINS 1932; VELLINGER 1929
Nile Blue 2B	—	LETORT 1932
Capri blue	—	do.
New Methylene blue	—	do.
Solid cotton blue	—	do.
Muscarine DH	—	do.
Viologens. Normal potentials, independent of pH		
Color in the reduced form		
Methyl viologen	—0.446	MICHAELIS and HILL 1933
Ethyl "	—0.449	do.
Betaine "	—0.444	do.
Benzyl "	—0.359	do.

in other connections, only those references which deal directly with the rH and pH as affecting rH of living material as found mainly through the use of dyes will be included. Although some examples have been reported (CHAMBERS 1933), indicating that not all electrometric measurements coincide with colorimetric determinations, or that even in a few cases measurements with indicators do not coincide, nevertheless it appears that these are exceptions rather than the rule. YUDKIN (1935) suggests that the reduction potentials recorded by indicators measure merely the "reduction equilibrium" of the dyes used, whereas the electrode potential indicates only that certain diffusible substances capable of affecting an electrode are in solution.

Relation of rH to pH. In all of these colorimetric and electrometric measurements, it is necessary to find the pH value in order to obtain the rH value, since acid-base systems and oxidation-reduction systems are intimately related. (See CLARK, et al. 1928.) Because protoplasm is a heterogeneous system there is considerable doubt as to whether one is justified in attributing pH value to it as a whole rather than to its various phases. Many of the differences in rH found by various investigators are due to the fact that different pH values were used in computing rH.

The two systems which are homogeneous are the solutions of cell suspensions and sap of *Valonia*. The experiments on cell suspensions will be more fully discussed later. These measurements are of the surrounding solution containing the metabolic products of the cells rather than of the cells themselves. In the case of *Valonia* sap, the solution which is being measured is nearly pure KCl (M. M. BROOKS 1926). The redox indicators must pass through the protoplasm before they enter the sap so that any change in the indicator is caused by the protoplasm as a whole, because the sap itself does not reduce the indicator. Moreover, such dyes as thionine and methylene blue (M. M. BROOKS 1929, 1930) are not chemically changed so far as could be detected by spectrophotometric analysis in their passage through the protoplasm into the sap. However, in computing rH value of the protoplasm of *Valonia* from the state of the indicators penetrating through it, the pH of the sap which bathes the protoplasm was used. This may prove to be incorrect. For the present, however, until such accurate determinations of the protoplasm itself can be made, this value has been used. In any case, if the pH of the protoplasm

should be around neutrality, as in *Amoeba* (CHAMBERS, POLLACK and HILLER 1927), this would at the shift the aerobic rH from around 18 to around 20.

Oxidation-reduction potentials

Natural Indicators. The oxidation-reduction potentials of a few natural indicators found in living cells have been determined.

PREISLER (1930) observed that the E_o' of the pigment of the nudibranch, *Chromodoris zebra*, is -0.102 volt, at pH 7.0.

CANNAN (1926) found that the pigment hermidin in the green plant *Mercurialis perennis* has an rH of 10. Since this plant is photosynthetically active, it was considered a striking example of the simultaneous low rH and the presence of molecular oxygen, indicating that molecular oxygen did not exert anything like its full theoretical potential in biological systems. The E_o' at pH 7.0 is -0.03 v. indicating at least 95% reduction. Another pigment which is found in the oxidized state in many echinoderms is echinochrome. CANNAN (1927) determined the E_o' at pH 7.0 to be -0.288 v.

The pigment pyocyanine which occurs in *Ps. pyocyanea* in the reduced form was found by FRIEDHEIM and MICHAELIS (1931) and FRIEDHEIM (1934a) to have an E_o' at pH 7.0 of 0.034 v. JUGLON and LAWSON, which are described by FRIEDHEIM (1934b) as accessory catalysts in respiration, were found to have an E_o' at pH 7.0 of $+0.033$ and -0.139 respectively. They are the coloring matter from husks of walnut and the leaves of henna plant (*Lawsonia inermis*) respectively.

The pigment of *Penicillium phoeniceum* was determined by FRIEDHEIM (1933c) to have an E_o' of 0.047 ; that of *Arion rufus*, (FRIEDHEIM 1933a) 0.025 ; and that of *Hallochrome* (FRIEDHEIM 1933b) 0.022 , all at pH 7.0. The red substance, 5,6-diketodihydroindole-2-carboxylic acid, which is intermediate in the formation of melanin, has also been found by FRIEDHEIM (1935) to have an E_o' at pH 4.62 of $+0.171$ v. STERN (1935) found a yellow pigment toxoflavin formed by *Bacterium bongkrek* to have an E_o' at pH 7.0 of -0.049 v. Cytochrome C from yeast has been found by COOLIDGE (1932) to have an E_o' at pH 7.0 of $+0.20$ v. The hemoglobin-methemoglobin system at pH 6.4, according to CONANT and FIESER (1925), has an E_o' of 0.217 v; BORSOOK, ELLIS and HOFFMAN (1937) found the redox potential of sulfhydryl

Table XXXV. Oxidation-Reduction Potentials
of Natural Indicators

Natural Pigment	E_0' at pH 7.0	Reference
Hemocyanin (<i>Limulus polyphemus</i>)	0.540 m. v.	CONANT, CHOW, and SCHOENBACH (1933)
Pigment of <i>B. phosphorescens</i>	0.280 (pH 7.6)	STONE and COULTER (1932)
Coenzyme-oxidase	-0.28	DEWAN and GREEN (1937)
Cytochrome C	0.260 0.250	COOLIDGE (1932) WURMSER and FILITTI-WURMSER (1938)
Hemoglobin-methemoglobin .	0.217 (pH 6.4) 0.152	CONANT and FIESER (1925) CONANT and PAPPENHEIMER (1932)
Pigment of <i>Penicillium phoeniceum</i>	0.047	FRIEDHEIM (1933, C)
Juglon	0.033	FRIEDHEIM (1934, B)
Pigment of <i>Arion rufus</i> . .	0.025	FRIEDHEIM (1933, A)
Hallochrome	0.022	FRIEDHEIM (1933, B)
Hermidin	-0.03	CANNAN (1926)
Pyocyanine	-0.034	FRIEDHEIM and MICHAELIS (1931), FRIEDHEIM (1934, A)
Toxoflavine (Pigment of <i>B. bongkreki</i>)	-0.049	STERN (1935)
Pigment of <i>Chromodoris zebra</i>	-0.102	PREISLER (1930).
Lawson	-0.139	FRIEDHEIM (1934, B)
Echinochrome	0.1995	CANNAN (1927)
Proteoflavine	-0.07	WURMSER and FILITTI-WURMSER (1937)
Lactoflavine	-0.208 0.185 0.187 0.188	BIERICH and LANG (1934) KUHN and BOULANGER (1936) BARRON and HASTINGS (1934) STONE and COULTER (1932)
Hepatonavine (Horse liver) .	0.188 (pH 4.13)	STARE (1935)

to be -0.390 ; FRUTON' (1934) found the potential of ascorbic acid at pH 7.0 to be -0.081 volt. The oxytocic hormone of the posterior lobe of the pituitary gland has been found by GULLAND and RANDALL (1935) to behave as an oxidation-reduction system with a potential of $+0.025$ v. at pH 6.0. Table XXXV summarizes these indicators according to the E'_0 scale.

Biochemical systems. In trying to determine the reasons for rH of cells and substances within cells, various reversible systems of biological significance other than indicators found in cells have been used in the study of oxidation-reduction potentials. Thus the potentials of reducing sugars, glucose, levulose, glucides and other carbohydrates have been studied by AUBEL and GENEVOIS (1926, 1928); AUBEL, GENEVOIS and WURMSER (1927); WURMSER and GELOSO (1928, 1929, 1931); BISHOP and WEST (1926); GOARD and RIDEAL (1924); PREISLER (1927); MAYER (1929); QUASTEL and WOOLDRIDGE (1929); MAYER-REICH (1934); The mechanism of the cysteine potential has been studied by KENDALL and NORD (1926); KENDALL (1928); KENDALL and LOEWEN (1928a, b); VOEGTLIN, JOHNSON and DYER (1926); JOYET-LAVERGNE (1927); MICHAELIS and BARRON (1929); MICHAELIS and FLEXNER (1928); BARRON, FLEXNER and MICHAELIS (1929); PREISLER (1930); DIXON and TUNNICLIFFE (1923); WARBURG and SAKUMA (1923); HARRISON and QUASTEL (1928); MASON, H. L. (1929); QUASTEL and WOOLDRIDGE (1929); QUASTEL and STEPHENSON (1926); GHOSH and GANGULI (1934); the alcohol-dehydrogenase-acetaldehyde system obtained from yeast and used by LEHMAN (1934); the lactate-succinate-pyruvate system investigated by DIXON and QUASTEL (1923); the lactate-enzyme-pyruvate system (BAUMBERGER, JURGENSEN and BARDWELL 1933); the lactate- α -hydroxyoxidase system measured by BARRON and HASTINGS (1934); the xanthine oxidase system by DIXON (1926); and the potentials of reduced carbohydrates in neutral and alkaline solutions as used by WURMSER and DE LOUREIRO (1934); dehydrogenase systems by GREEN and DEWAN (1937). An adequate review is given by MICHAELIS and SMYTHE (1938). A summary is given in Table XXXVI.

Cell suspensions

The method of suspending bacterial cells and measuring the potential by means of an electrode was first used by GILLESPIE

Table XXXVI. Oxidation-Reduction Potentials of Biochemical Systems

Redox System	E_o' at pH 7.0	Reference
Cystine-cysteine	—0.222 m.v. —0.390 —0.329	FRUTON and CLARK (1934) BORSOOK, ELLIS and HUFFMAN (1937) GHOSH, RAYCHAUDHURI, and GANGULI (1932)
Glycylcystine-glycylcysteine .	0.025	GREEN (1933)
Glutathione-reduced glutathione	0.068 0.062	GHOSH and GANGULI (1935) GREEN (1933)
Dithiolactic acid-thiolactic acid	—0.321	BORSOOK, ELLIS and HUFFMAN (1937)
Succinic-fumaric acid. . . .	—0.020	STOTZ and HASTINGS (1937)
α -hydroxyglutaric- α -ketoglutaric acid . . .	—0.070	WEIL-MALHERBE (1937)
Ascorbic acid	—0.081	FRUTON (1934)
Succinic- maleic acid . . .	—0.102	LAKI (1935)
Lactic- pyruvic acid	—0.150	BARRON and HASTINGS (1934)
Malic- oxalic acid	—0.169	SZENT-GYÖRGY and LAKI (1937)
Malic- oxalecetic acid . . .	—0.169	LAKI, STRAUB, and SZENT-GYÖRGY (1937)
β -hydroxybutyric-acetoacetic acid	—0.282	GREEN, DEWAN, and LELOIR (1937)
Xanthine- uric acid	—0.361	GREEN (1934)
Hypoxanthine- uric acid . .	—0.371	GREEN (1934)
Adrenaline	0.390 (pH 6.8)	BALL and CHEN (1933)
Catechol	0.385 (pH 6.8)	BALL and CHEN (1933)

(1920). He placed an electrode of an indifferent metal in a medium of living organisms and observed an orderly drift toward highly negative potentials of high reducing intensity. Since then many experimenters have used this method to measure the reducing

intensity of suspensions of organisms. It must, however, be kept in mind when measuring the potential of suspensions of living cells, that the medium in which the cells are suspended is being measured and not the cells, as was pointed out by MICHAELIS (1929). The surrounding solution of course includes substances which diffuse out of the cells under certain conditions, and gives an index of the changing state of the cell itself. There is, therefore, a definite value in the results obtained by this method, although absolute values for the state of the cell itself cannot be claimed.

CANNAN, COHEN and CLARK (1926) measured suspensions of yeast cells by the same methods, and found the pH to be 6.8 under aerobic conditions and the rH within the indophenol region. The gross reducing intensity was stabilized between +0.1 and +0.2 volt at pH 7.0. Under anaerobic conditions, a general drift to highly negative potentials was produced. They also found that suspensions of various cells, including *B. coli*, *B. fluorescens*, *B. subtilis*, *Streptococcus* and minced rat liver are poorly poised with respect to electromotively active material present at any moment, but that active material is slowly mobilized by cell catalysis from some large reserve. This causes a gradual orderly drift toward highly negative potentials with the course of time, and is indicative of high reduction intensities as was first observed in cultures of bacteria by GILLESPIE (1920).

HARVEY (1929) found the rH of luminous bacteria in an aerobic state to be between 18 and 20, and in an anaerobic condition to be between 8 and 10.

AUBEL and GENEVOIS (1927) also measured suspension of yeast and *B. coli* by electrometric and colorimetric methods and obtained consistent measurements. Under anaerobic conditions the rH of yeast suspensions was 7, while that of *B. coli* was much lower. These values are somewhat lower than those of CANNAN, COHEN and CLARK (1926).

BOZA and REED (1931) found low reduction potential in cultures of *E. coli* under anaerobic conditions.

From the point of view of permeability, this means that the plasma membrane is permeable both to substances which have a low reducing intensity under anaerobic conditions, and to substances having a higher electrode potential under aerobiosis.

AUBEL, AUBERTIN and MAURIAC (1928) used cell suspensions of mammalian tissues. There is an error due to cytolysis by grind-

ing up tissues. They found an rH of 20 in aerobiosis and an rH between 11 and 12 in anaerobiosis. VOEGTLI, JOHNSON and DYER (1924) used thin slices of a number of different tissues from the rat, staining them under anaerobic conditions. They also injected these dyes intravitaly and determined the range of the indicator scale on which reduction took place. Since some cytolysis must have taken place on the surface of the injured tissues, as pointed out by the NEEDHAMS (1925) this would tend to lower the rH value in each instance producing a systematic error. Their results show that reduction takes place with certain indicators. The time necessary to reduce an indicator at different concentrations by a given tissue is also given. However, this method would necessarily have to be only roughly quantitative, as CLARK points out (1928), and it would not resolve the time required into its two components, namely, that required to reach definite potentials with the material naturally at the disposal of the cell suspension, and that required to overcome the poisoning action of the added indicator. This probably explains the discrepancies regarding the effect of dye concentration found in this work with that of AHLGREN (1925). Since the capacity factor involves an exact quantitative method which at the present writing is only roughly so, any results accruing from this method must be necessarily be given with this reservation.

When minced tissues are used *in vitro*, the effects of cytolysis produce complications which result in abnormal conditions and usually lowered readings on account of the formation of highly reducing substances produced by injury as previously mentioned (HAMMET 1929). Nevertheless, a number of investigators have used these methods.

AUBEL, MAURIAC and AUBERTIN (1929) mention two causes of error; bacterial contamination when cultures are exposed for several days, and autolysis.

Relation of rH of Medium and Growth. The redox potential of sterile culture medium has been studied by SMITH (1896); COULTER (1928, 1929, 1930); LEPPER and MARTIN (1930); KNIGHT (1930); BOYER and REED (1931); DUBOS (1929) who show that there is present an active oxidation-reduction system.

AUBEL and AUBERTIN (1927) found that the growth of facultative and of strict anaerobes took place only in media which had a definite reduction potential and concluded that the rH

of the cells was in equilibrium with the rH of the medium. For example, *B. botulinus* grows only in medium which has an rH of 12. They concluded that anaerobic bacteria can live only between rH 0 and 12, while facultative anaerobes can exist between rH 0 and 20. AUBERTIN, AUBEL and GENEVOIS (1928) have further found that strict anaerobes could be cultured in the presence of O_2 provided that some highly reducing substances as pyruvic acid or cysteine were added to the culture medium.

QUASTEL and STEPHENSON (1926) found that the presence of small amounts of cysteine or other —SH compounds will induce good aerobic growth of *B. sporogenes*, an anaerobe. They believe that cysteine establishes a definite limiting reduction potential necessary for growth.

COULTER and ISAACS (1929) found that the E_h of *B. typhosus* is stabilized when O_2 is passed through continuously and does not show a negative drift, but that there is a negative drift under other circumstances. They concluded that reduction substances of bacterial origin, at least in the typhoid bacillus, do not influence the electrode potential in the presence of O_2 . Further studies on the relation between the growth of anaerobic bacteria and the reduction potential of the surrounding medium were made by REYMANN (1928) and DUBOS (1929). BOYD and REED (1931) measured cultures of *E. coli* in different solutions.

Experiments in which the medium was poised by the addition of reducing or oxidizing agents were performed by BROWN and BALDWIN (1933) who influenced the growth of aerobic bacteria by adding small concentrations of reducing or oxidizing agents to the medium. KNAYSI and DUTKY (1933) showed the effects of free oxygen on the growth of *B. megatherium* at different potentials. The same authors (1934) conclude that the O_2 content of the medium and not the oxidation-reduction potential is responsible for growth. INGRAHAM and FRED (1933) state that the bacteriostatic action of gentian violet is a function of its poisoning capacity rather than of its ability to form a dye-protein compound in the bacterial cell. REISS (1930) concluded that the development of sea urchin and *Sabellaria* eggs depends upon the oxidation-reduction potential. (See also RAPKINE 1929).

MCCARTER and HASTINGS (1935) were able to produce either smooth or rough type of growth of an acid-fast organism by changing the redox potential of the medium.

CLIFTON (1933) found that *Staphylococcus aureus* develops and maintains rather intensive reducing conditions. After lysis by a specific bacteriophage, the potential becomes more negative. CLIFTON, CLEARY and BEARD (1934) have measured the redox potentials of the medium and that of the organisms growing in it by using a continuous flow of peptone and conclude that there is a marked fall of potential during the period of rapid growth, and a maximum reduction potential is developed in or near the maximum stationary growth period. In glucose-peptone cultures CLIFTON and CLEARY (1934) found a more marked fall in potential and a higher maximum in the viable count during the period of rapid growth. They conclude that the observed redox potentials in bacterial cultures are a resultant of the metabolic activities of the cells.

BURROWS and JORDAN (1934) found that different species of *Salmonella* cultures produced potentials in the media which differed from each other, suggesting that these potentials were due to the bacterial substances synthesized. MESSING (1934) has used sulfhydryl compounds to promote the growth of anaerobic bacteria under aerobic or anaerobic conditions and comes to similar conclusions as CLIFTON, et al. (1934). ZOBELL (1935) adjusted the redox potential of the medium in which marine nitrifying bacteria were grown and found that no nitrite was formed when the potential was lowered. YUDKIN (1935) measured the reduction potentials of *B. coli*, *alcaligenes faecalis* and *Cl. sporogenes* both electrometrically and colorimetrically with indicators. He found that the potentials by both methods agreed fairly well. Addition of succinate did not lower the potential, but glucose and formate induced more negative potentials.

MAHONEY and TAYLOR (1930) found that certain oxidation-reduction indicators rendered diphtheria toxin atoxic.

Relation of O_2 Tension to rH and Growth of Organisms. COULTER (1928), extending the observations of SMITH (1896), has determined electrometrically the potential of sterile bouillon, withdrew the oxygen and noted that the potential was dependent upon the oxygen present and not on the activity of living cells in that case. Using a culture of *B. typhosus*, COULTER and ISAACS (1929) passed O_2 through continuously and did not observe a negative drift, such as was obtained, for example, by GILLESPIE (1920) and by CANNAN, COHEN and CLARK (1926). The E_h remai-

ned between -0.085 and -0.095 in an atmosphere of O_2 . They attribute the potential to the hypothesis that reductive substances produced by bacteria do not influence electrode potentials in the presence of O_2 . They also call attention to the fact that all investigators have noted that reduction potentials of considerable intensity are attained only when O_2 is removed from the system, whether this removal is accomplished by deaeration by a stream of nitrogen or by spontaneous O_2 consumption. Removal of O_2 by respiration of the bacteria is sufficient to explain the attainment of this level of reduction potential in a growing culture and therefore the writers state that it is this factor and not the elaboration of reductive products by living bacteria which is responsible for reduction potentials. The immediate response of the electrode to small amounts of O_2 further emphasize this view. They state further that only when bacteria die is reductive material liberated, and when O_2 is passed through continuously, reductive substances do not influence the electrode potentials in the case of *B. typhosus*.

On the other hand, KUSNETZOW (1931) states that the fall in potential of the nutritive medium containing *Aspergillus niger* takes place in consequence of the evolution by the organisms of some kind of stabile substance possessing strong reductive capacities.

In the case of COULTER and ISAAC's studies, as they themselves state, it is not possible to distinguish the effect of O_2 on the electrode from the true electrode potential which is found in the absence of O_2 .

QUASTEL and WOOLDRIDGE (1929) have contended from experiments on *B. coli* which were placed in lactate-succinate-fumarate media, that the growth of the cell does not depend upon rH. When relatively high concentrations of these substances were used, however, there was an effect on growth, showing that it is necessary to "poise" the medium to show the effect. It has been pointed out by CANNAN, COHEN and CLARK (1926) that "appreciable quantities of dye bring into play an extraneous capacity factor" which poises the solution at a definite rH. It seems, therefore, in the case of the experiments of QUASTEL and WOOLDRIDGE that the medium they used was not sufficiently poised to produce the desired rH when small concentrations of substances were used, but that amounts sufficient for poisoning the potential did produce an inhibiting effect.

LEHMAN (1929) noted that there was a cessation of the drift in potential in a succinodehydrogenase solution on the addition of methylene blue; and RAPKINE (1929) was able to cause normal development of sea urchin eggs when the rH of the solution was poised by methylene blue.

DUBOS (1929) used *Pneumococcus* and *Streptococcus* in his oxidation-reduction studies with indicators, and concluded that the medium is poised outside the range in which the organisms grow when inhibiting dyes are used.

HEWITT (1930) investigated the redox potentials of *Pneumococcus* cultures and found them similar to those of *Streptococcus* but different from diphtheria and *Staphylococcus*.

HEWITT (1932) added catalase to the medium in which hemolytic *Streptococcus* was growing and noted that the electrode potential falls to a lower level under aeration.

KNIGHT and FELDES (1930) found that the germination of *Cl. tetani* was completely inhibited at a potential more positive than E_h of 0.11 volt at pH 7.0 to 7.65.

The relation of the oxidation-reduction potential to the growth of an aerobic organism, *B. megatherium*, was studied by WOOD, WOOD and BALDWIN (1935). They found that indicators negative to methylene blue in the electromotive series fail to inhibit growth. However, those compounds which inhibit growth in the oxidized form are bacteriostatic when reduced.

FREI and RIEDMULLER (1931) have found that anaerobic growth is affected by the oxidation-reduction potential of the medium.

In order to see whether the constant presence of O_2 affected the rH value, RAPKINE and WURMSER (1926) (See also CANNAN 1926 p. 3), microinjected *Spirogyra* in the process of photosynthesis, and found the pH to be 6.2 and the rH 14.4 to 16. The low potential suggests that the presence of molecular O_2 is not the limiting factor in determining rH but that O_2 must be activated for oxidation-reduction phenomena.

RAPKINE (1929) has been able to cause sea urchin eggs to develop normally by placing them in a closed tube containing boiled sea water to which has been added a small amount of methylene blue; whereas those which had no dye did not develop. The rH of the medium was poised by the dye enabling the eggs to develop. LYON and SHACKELL (1910) showed that the rate of staining of living fertilized sea urchin eggs by methylene blue

and dahlia was more rapid than that of unfertilized eggs. This would indicate a greater reducing activity on the part of the fertilized eggs or lesser permeability on the part of unfertilized eggs. In either case the visible effect would be the same, namely, a paler color in the interior of the egg.

REISS and VELLINGER (1929) also found that the eggs of the sea urchin were capable of developing in the absence of free O_2 provided that there is a certain redox potential maintained by adding hemoglobin, hemocyanine or certain dyes.

CONANT (1926) states that the mere addition of O_2 has no relation to the O_2 potential. CANNAN (1926) using the pigment, hermidine of the green plant, *Mercurialis*, also concluded that molecular O_2 does not exert anything like its full electrode potential in biological systems.

It is of interest to note that HARVEY (1927, 1929) found that the most positive dyes are reduced rapidly even before luminescence stops; the most negative only after it stops due to the utilization of O_2 by bacteria. In the case of suspension, CLARK (1925) never finds the E_h more positive than about 0.2 volt at pH 7.0 as measured by both indicators and electrode, even after air has been bubbled through the suspensions, while certain anaerobic bacteria can induce the potential of the hydrogen electrode and also produce a slight overvoltage.

The question then arises, what rôle does molecular O_2 have *per se* in producing the electrode potential which corresponds to a minute fraction of the ordinary O_2 pressure found in air? Yet when the external O_2 pressure is lowered below normal, the E_h is lowered; and when the O_2 pressure is raised above that found in air the E_h is not changed.

If we take the rH at 20.5, for example, the hypothetical oxygen pressure in the cell is 10^{-41} atmospheres, almost a negligible quantity. While the rH represents this condition in the cell under aerobiosis, the pressure of O_2 in the surrounding solution may be considered to be 2×10^{-1} atmospheres (that in air). The rH then represents a minute fraction of the total O_2 pressure of the environment in which the cell lives. This fraction is the electromotively active system. An increase in the O_2 pressure above that in air does not increase the rH of the cell, but a decrease beyond a certain limit causes a decrease in the rH of the cell, so that even smaller and more hypothetical values are obtained.

In some of the experiments reported the rH values have been determined under two sets of conditions, those in which the organisms were kept in air or oxygen, and those kept under anaerobic conditions. The results are designated as aerobic rH and anaerobic rH . Both values are of such a low order of magnitude as to be almost hypothetical, but the latter values are even lower than the former. They are interpreted thermodynamically as referring to the negative logarithm of a hypothetical hydrogen pressure. The interpretation of this in terms of the presence of oxygen is rather vague and can be designated as that "activated" proportionate concentration of oxygen out of the total concentration of oxygen present, which includes the "activated" and the molecular. And finally we must consider the effects of molecular oxygen itself on the metallic electrode making the measurements designated as oxidation-reduction potentials.

If the organism could be conceivably regarded as existing in the complete absence of molecular oxygen, then the rH would be interpreted as the total oxygen present. In this case the O_2 would be 100% "activated" or electromotively active. This then, means that an inconceivably small fraction of oxygen is used in this state by the organism. With our ordinary methods of producing a vacuum this state of anaerobiosis is probably not attained. Since the rH obtained under aerobic conditions is still of a very small order of magnitude, although greater than the anaerobic rH , it still means that a minute fraction of the oxygen available is the maximum concentration used in the "activated" condition by an organism, and that the large proportion of molecular oxygen is not available and is not being used.

It is of interest that the presence of free molecular O_2 , as for example, that produced by photosynthesis in plants, does not give rH values in the region of O_2 overvoltage as would be expected if all the O_2 were "activated". It is only possible to conclude, therefore, that a very small proportion of the O_2 is in the "activated state". It is difficult to formulate ideas on the utilization of O_2 or its method of "activation" at present. The relation between electrode potential and activation of H_2 or O_2 involves a rearrangement of atoms and consequent shift in electron bonds. And finally the term rH , whether it proves in the last analysis to have any real value, is for the present a convenient scheme employed as a "scaffold for obtaining working equations". To

quote CLARK (1928), "it must be said that while there can be no valid objection to the method of presentation which employs concepts of electrodes acting as hydrogen or oxygen electrodes, it has had the unfortunate effect of leading many investigators to the belief that experimental confirmation of derived relations proves the actuality of the mechanism postulated".

Interior of Cells. In order to ascertain the oxidation-reduction potential of the interior of living cells, various methods have been used.

The first experiments in which oxidation-reduction dyes were systematically applied in biological experiments and rH values obtained were those of J. and D. NEEDHAM (1925), who used the microinjection method with protozoa. The second experiments were those of M. M. BROOKS (1926), who used the immersion method and determined whether or not the dye penetrating into the sap of the marine alga *Valonia* was in a reduced or oxidized state. Following these experiments, a number of investigations have been made on different plants and animals, parts of tissues, cells, and of media under various conditions of aerobiosis, anaerobiosis, pH values and temperatures. Several reviews have been made of this literature, to which the reader is referred (MICHAELIS 1933, CHAMBERS 1933, MICHAELIS and SMYTHE 1938). In the present discussion only those references which seem to have a bearing upon permeability will be considered.

Methods of measuring. Microinjection. The microinjection method as perfected by CHAMBERS (1922) has been widely used in comparing the state of dye which was injected into the interior of a cell with that which has penetrated from the outside.

PLOWE (1931) states that no injury to the cell is produced by inserting micropipettes into living cells. KOPAC (1935) states that probably the reason for this is that the inserted object is immediately walled off by a layer of protoplasm covering it and separating it from the rest of the cell. J. and D. NEEDHAM (1925) first determined the pH by microinjection into *Amoeba* solutions of neutral red, phenol red and bromthymol blue and obtained a value of 7.6. Then they microinjected the oxidation-reduction indicators and from these results calculated the rH to be between 17 and 19. These results have been subsequently criticized by various workers on the basis of the method of ob-

taining the pH and on their method in using the oxidation-reduction indicators. VLÈS (1926) and later POLLACK (1928) state that too much phenol red was injected in determining pH, thereby masking the true color of the protoplasm. REISS (1926), RUMJANTZEW and KEDROWSKY (1926) and the NEEDHAMS (1925) have further called attention to the fact that neutral red stains only inclusions and not the protoplasm of *Amoeba*, thus giving an incorrect value when used as a protoplasmic stain. (See also GUILLIERMOND 1927). This also applies to PANTIN's results (1923) on the microinjection of neutral red into *Amoeba*. In the case of NEEDHAM's results (1925) since cytolysis took place shortly after the organisms were injected, the animals were evidently not normal when the readings were made. When *Amoeba* cytolyzes it liberates very vigorously reducing substances during the death of the cell, as PANTIN (1923) and the NEEDHAMS (1925) have found when they were able to cause the reduction of neutral red under these conditions.

Since determinations of rH values are dependent upon pH values, it can be readily seen that variations in results in which pH values were determined would necessarily result in differences in rH, even in the same animal. This is found to be the case in a number of experiments.

SCHMIDTMANN (1924, 1925) also attempted to obtain the pH of tissues by injecting minute granules of indicator dyes into tissue cells obtained from teased preparations and from frozen sections. The color produced by the dissolved granule was compared with that resulting from a similar granule in a deposit of a known buffered solution. Both vertebrates and invertebrates were used and a considerable variation was found in the pH of different cells. In general SCHMIDTMANN concluded that the majority of the epithelial cells in mammals are almost neutral or slightly alkaline (pH 6.9—7.5), while muscle and connective tissue tend to be slightly more acid (pH 6.6—6.8) and capillary endothelium most acid of all (6.3—6.4). SCHMIDTMANN noted that the color of the injected dye tended to diffuse from cell to cell. This performance is commented on by CHAMBERS (1929) as being probably due to injury of the tissue, as he found that the teasing of tissue has to be most cautiously performed in order to obviate injury, and that freezing was always fatal to the cell. The high concentration of dye which would necessarily result on

introducing a solid particle no matter how small, would cause the color of the dye to mask the true color as affected by the protoplasm.

KOPAC (1935), with refined technique, has injected the various pH indicators into different marine eggs to ascertain pH values. He found that most of the indicators diffused readily through the cytoplasm, even though these indicators are known not to penetrate readily from the outside, and that most of them readily stained the astral and spindle zones of ascidian eggs, whereas in sea urchin eggs the formation of an internal membrane at the site of injection of the dye apparently prevents diffusion.

In order to see whether the rH was poised in these organisms, the NEEDHAMS (1926) injected into *Amoeba* the reduced form of a dye lower in the scale than the one which is partially reduced by the organism and noted that the color returned to its oxidized form. They concluded from this that the cell has a definite well-poised characteristic equilibrium. By subjecting *Amoeba* to atmospheres of O₂, N₂ and H₂ the NEEDHAMS found no differences in the rH. This result is not in agreement with the subsequent results of COHEN, CHAMBERS and REZNIKOFF (1928), who avoided some of the early pitfalls and found the rH under anaerobic conditions to be 7.5 at pH 7.0; under aerobic conditions, the reducing intensity ranges from rH 13 to 18, depending on surrounding circumstances and showing the same lack of poisoning ability of this system as was found with cell suspensions. Further refinements in the method showed that *Paracentrotus lividus* and *Amoeba proteus* gave an rH of 12 under aerobic, and of 9.2 under anaerobic conditions, or —0.07 volt and —0.143 volt respectively at pH 7.0 (CHAMBERS, COHEN and POLLACK 1932).

It is possible that some of the differences may be explained by the lack of agreement on the part of these two sets of investigators as to the pH of *Amoeba*. The NEEDHAMS considered the pH to be 7.6, while COHEN, CHAMBERS and REZNIKOFF (1928) used 6.9, the value found by CHAMBERS, POLLACK and HILLER (1927). VLÈS (1926) has further commented, if the NEEDHAMS had corrected for salt error, protein error and error of the indicator, their pH value would be considerably lower, and in that case would approach that found by CHAMBERS, et al. However, since these errors of the protoplasm are unknown and would have to be merely

conjectures, it is difficult to see how such corrections were to have been made.

The NEEDHAMS (1926) also microinjected a variety of marine eggs and found the rH to vary between 19 and 22 and the pH to be 6.6. This latter value is also criticized by VLÈS (1926) on the above grounds. They also used *Nyctotherus oviformis* (1926) aerobically and anaerobically in an atmosphere of H_2 , N_2 and O_2 respectively and found that the rH changed from between 19 and 20 under aerobic conditions to between 9.5 and 10.5 in anaerobic atmosphere. This differs from their results with *Amoeba* (NEEDHAMS 1926) in which they found a definite poisoning equilibrium.

CHAMBERS, POLLACK and COHEN (1929), injecting CLARK's indicators, found that the rH of starfish eggs and of sand dollar eggs under aerobic conditions to be around 12, and the anaerobic rH 7.9. These figures are lower than those obtained by the NEEDHAMS (1926) and RAPKINE and WURMSER (1927) for marine eggs. They concluded that the reduction potential in aerobic cells studied is a function of the presence or absence of O_2 in the environment.

Various other animal materials have been used: the nucleus and cytoplasm of cells of the salivary gland of *Chironomus* and *Calliphora* larvae by RAPKINE and WURMSER (1926); the blastocoele of sea urchin by RAPKINE (1927) who found the rH to be between 19 and 19.6. CHAMBERS and POLLACK (1927), on the other hand, found the rH to be the same as that of the outside solution, showing an intimate relation between the vacuole and the external environment.

The eggs of *Paracentrotus lividus* and of *Asterias rubens* under aerobic conditions were found by RAPKINE and WURMSER (1926) to have a pH of 7.2 and an rH of 19 to 20.4 in all cases. REISS (1926), however, thought that these values were too high, being dependent on too high a pH value (7.0 to 7.3), and states that the real pH value was masked by the use of too concentrated indicator solutions. However, RAPKINE and WURMSER (1928) stated that the dyes were reduced at too low a pH to affect the experiments.

AUBEL and LEVY (1929) state that the rH was dependent upon the presence or absence of O_2 . They microinjected larvae of *Galleria mellonella* and *Phormia regina* and obtained an rH

of > 20 in air and 7 in N. They also (1930) microinjected living slugs and found the rH to be 20 under aerobic conditions and 5 to 6 under anaerobic conditions.

RAPKINE and WURMSER (1926) microinjected *Spirogyra* during the process of photosynthesis, and found the pH to be 6.0 ± 0.2 and the rH 14.4 to 16. This value for plants is lower than that found by M. M. BROOKS (1926) for *Valonia* in direct penetration experiments and also lower than that found for most animals by other workers.

The results obtained by the microinjection method are somewhat varied, as would be expected when the pitfalls of a new method were not fully evaluated. The various values for rH under aerobic conditions however, seem to group themselves mainly around 17 to 20, and under anaerobic conditions around 7, indicating that there is some effect of the presence or absence of molecular O_2 .

Direct Penetration. The NEEDHAMS (1926) placed fertilized and unfertilized echinoderm eggs in solutions containing o-chloro phenol indophenol, 2,6-dibromo phenol indophenol, o-cresol indophenol, and o-cresol dichloro phenol indophenol, and the indigo sulfonates. They found that the first four dyes and those most easily reducible stained the eggs, while the sulfonates or those most difficult to reduce did not. There seems to be no question that the dye which they used was too concentrated (1%) and injured the cells, so that the dye penetrated in the oxidized state; whereas the indigo sulfonates did not show color because they did not penetrate. When they microinjected the dye into the eggs they found that the ones which were easily reducible were reduced in the cells. This latter result agrees in general with the immersion experiments by M. M. BROOKS (1926) on *Valonia*.

Other methods used by the various workers are subject to the criticism that injury has been produced by subjecting the plant to abnormal conditions. MARTIN (1927) and SMALL (1926) have attempted to find the H-ion concentration of a variety of plant tissues by cutting sections from various regions, soaking them in distilled water and then placing them in indicator solution overnight and observing the color at various places in the tissue under the microscope. The injury produced by this method is recognized by the authors, and the pH values which they obtain

are in all probability too low. No method has yet been devised for staining normal protoplasm with acid-base indicators. Nor is the pH of sap obtained by crushing tissues and extracting the sap normal owing to chemical changes, as has been frequently mentioned in the literature.

The first study on the penetration of these indicators into plant cells is that of M. M. BROOKS (1926, 1927, 1929, 1930, 1931, 1932) who worked with the marine alga, *Valonia*.

Valonia is a large coenocytic marine alga, consisting of a central vacuole filled under osmotic pressure with colorless sap which has been analyzed by MEYER (1891), WODEHOUSE (1917), OSTERHOUT (1922) and S. C. BROOKS (1930) and found to contain mostly KCl and a small amount of NaCl. A delicate layer of protoplasm surrounds the sap and this is enclosed in a cell wall of cellulose (SPONSLER 1931). The sap comes out readily and uncontaminated when a tiny cut is made in the wall and protoplasm. The amount of sap varies up to 50 cc. *Valonia macrophysa* J. G. AGHARD, is smaller and usually branched, while *Valonia ventricosa* KÜTZ is found as separate cells.

In using *Valonia* the advantage lies in the fact that the sap which is contained inside the protoplasm is an indicator, so to speak, of what passes through the protoplasm of an intact, living, normal cell; that the sap is a homogeneous solution which can, therefore, be correctly considered as possessing certain pH values or rH values; that the sap is practically unbuffered and is, therefore, an accurate indicator, with certain limitations, of certain physiological properties of the living, unaltered protoplasm.

In the experiments of M. M. BROOKS *Valonia* plants were placed in solutions of CLARK's indicators for a definite time after which the sap was extracted without contamination, and direct colorimetric and spectrophotometric determinations were made. The indophenols which are easy to reduce, penetrated readily and were found in a reduced form in the sap. The color was brought back by an oxidizer. The thiazines which are reduced with greater difficulty and are more electronegative than the indophenols penetrated readily but were found oxidized in the sap. The indigo sulfonates, which are the lowest on CLARK's scale and the most difficult to reduce, did not penetrate.¹⁾ (See section on sulfo-

¹⁾ Indigo tetrasulfonate was found in the sap in a yellow form but this has not been further studied. Since this substance would not be either

nated dyes, Chapter XII.) There was no toxic action of these dyes in *Valonia* with the concentrations used during the time indicated.

BECKER (1926) suspended the protozoa, *Opalina ranarum* and *Paramoecium*, in solutions of janus green in absence of air and found that the granules which took up the dye were capable of reducing it. Since the pH of the granules was not determined, the rH could also not be calculated. Quantitative values were not obtained

Further experiments with neutral red and janus green which have an electrode potential more negative than any of CLARK's dyes (RAPKINE, STRUYK and WURMSER 1929) show that the electrode potential is not the factor which determines whether or not a dye penetrates living cells, but it does determine whether or not a dye is reduced by the organism. (See also M. M. BROOKS 1926, and BARRON and HOFFMAN 1930.)

Following these results, CHAMBERS, COHEN and POLLACK (1931, 1932) and CHAMBERS, BECK and GREEN (1933) placed eggs of the sand dollar and the starfish into solutions of these indicators and noted which dyes were reduced by the suspensions. They showed essentially the same conclusion which the NEEDHAMS (1925) found for protozoa, which BROOKS (1926) found for *Valonia*, and which VOEGTLIN, JOHNSON and DYER (1924)¹ found for tissue lice, namely, that all dyes above a certain potential are reduced by the suspensions and those below remained oxidized. The turning point, however, in the case of the experiments of CHAMBERS, et al., was lower than for *Valonia*, indicating a greater reducing intensity in the case of the eggs. Since the results obtained by CHAMBERS, et al., with suspensions of eggs agree fairly well with those obtained by him with the microinjection method, the lower values may be attribute in both cases to the formation of substances not normally found in the cells but produced by the methods of experimentation; in the former case, the injury of microinjection, and in the latter case, the presence of metabolites in the suspension, which was being measured. (See section on cell suspensions.)

oxidized or reduced by any of the ordinary methods, it appears that irreversible chemical alteration of the dye had taken place.

¹) While these values are undoubtedly systematically too low, owing to the method used, nevertheless they show a progressive increase in reducing intensity.

It has been found that reduced dyes of a certain potential become oxidized in living cells (NEEDHAM and NEEDHAM 1926). ROSKIN and MASLOWA (1935) show that hyposulfite white of neutral red and methylene blue penetrated into frog mesentery, white mice mesentery, the nucleus of red blood cell and onion cells and became oxidized inside the cells.

Intravital Injection. A fourth method which has been used for determining pH and rH of tissues is that of injecting oxidation-reduction indicators intravenously into living animals, with subsequent killing of the animals and analysis of the color in various tissues. This method has been used by FRIEDHEIM (1929) for mammalian tissues and embryo juice, by JAUMAIN (1925) in determining the rH of the blood, by REISS (1926) for serum, and for mammalian tissues and blood by MAURIAC, AUBERTIN and AUBEL (1928).

AUBEL and WURMSER (1929) by the same method found the rH of stomach, intestine and pancreas between 16 and 20; that of muscle and kidney between 14 and 16; that of brain and liver about 9. VOEGTLIN, JOHNSON and DYER (1924) used this method first in one of the earlier experiments, but did not estimate the rH.

Since the tissues in some of these experiments were sliced for analysis, the injury so produced would presumably lower the rH value by liberation of reducing substances.

Crushing and Freezing. Believing that the microinjection method injured the cell (PÉTERFI 1927)¹, VLÈS, REISS and VELLINGER (1924), VLÈS (1924) used a third method for determining the pH and rH of cells. They froze sea urchin eggs to -60°C. , pounded the mass of eggs while frozen in a mortar to prevent the escape of CO_2 , and then took electrometric measurements of the thawing mass at 0°C. , estimating the pH to be between 5 and 6. Using the same method of freezing, REISS and VELLINGER (1926) found the pH of frog muscle to be between 5.9 and 6.2, and that of mouse muscle between 6.0 and 6.2.

VELLINGER (1926a) found the pH of *Paracentrotus* eggs to be 5.8 to 5.9 and that of *Arbacia* to be 5.0 to 5.2. These results agree with the pH value found by VLÈS (5.8, 1924) for *Paracentrotus*

¹ PÉTERFI (1927) working with myoblasts and monocytes found that a thixotropic sol-gel transformation occurs on microinjection which is typical and different from every cell investigated.

when the same method of freezing was used in combination with the colorimetric analysis. The rH value of sea urchin eggs was put at 20.6 by VELLINGER (1926b). VLÈS (1926a) also found the pH of potato by the same method of freezing, crushing and thawing when corrections for salt and protein errors and error of the indicator were made.

As a general criticism of this method, it may be stated that the alternate freezing and crushing of the cells and tissues, even though preventing the escape of most of the CO_2 , would bring together foreign substances by breaking down normal morphological barriers, intermingling different phases, whereby chemical reactions would produce abnormal conditions. Cytolysis plays an important role in increasing the pH by liberation of abnormal amounts of such substances as acids or the products of high reducing intensity (such as cysteine found by HAMMETT (1929) in roots which had been crushed). A comparison of the values for pH obtained by this method with those obtained by other methods shows that they are considerably lower, and that they must be interpreted with these facts in mind.

Oxidation-reduction Potentials of Parts of Tissues and Cells.

REDSLOB and REISS (1930) found the rH of vitreous humor to be 19.1 at pH 7.6 using both electrometric and colorimetric methods. FRIEDHEIM (1929) states that the rH of cancerous tissues is no less reducing than that of normal.

JOYET-LAVERGNE in a monograph (1933) and subsequent work, concludes from a series of determinations of various plants and animals, that there is a difference in the reducing intensity of tissues depending upon the sexuality of the tissue or cell. This seems rather a startling conclusion when one considers the heterogeneity of protoplasm and the concept that the rH of such a system is a loose term at best.

ALEXANDROW (1932) found that the larva of *Chironomus plumosus* kept in water saturated with O_2 gave an intracellular rH of the tissues of > 15 ; in anaerobic conditions, of 7.7.

LEWIS (1935) found that fluorescent X which is reduced neutral red (CLARK and PERKINS 1932) was taken up by living tissue cultures and accumulated in certain granules as the reduced yellow form and became oxidized to neutral red.

BECKER (1932) has shown the influence of the oxidation-reduction dyes of the azine series on somatic mitoses in the roots

of *Allium cepa*. He found that solutions of thionine, toluidine blue and cresyl blue show abnormal nuclear division when the roots are grown in them, whereas janus green, neutral violet safranine and phenosafranine are much less active in causing abnormalities. In the first group the rH is from 14 to 15; in the second, from 4 to 7. He suggested that the difference is due to the oxidation-reduction potential of the two sets of dyes.

A number of experiments have been done showing whether or not some of the oxidation-reduction dyes penetrate living cells without determining the rH or their comparative reducing intensity. Since no simultaneous pH readings were given this cannot be calculated. FAURÉ-FREMIET (1923) states that the egg of *Sabellaria* is permeable *in vivo* to neutral red, sulfate and chloride of Nile blue, and brilliant cresyl blue. Details as to what parts of the eggs are stained were not given.

MANGENOT (1928) states that cresyl blue penetrates into the cells of certain algae, combines with KI to give an oxonium compound of I which is then deposited as red crystals.

The rH of the cilia of gill tissue of *Pecten yessoensis* was studied by NOMURA (1933). He stained pieces of gill tissue with a series of the oxidation-reduction indicators. He considered the critical potential at which ciliary movement stopped to be that of the rH of the indicator which was reduced at the time of cessation of movement. No mention is made of what parts of the cell were stained by the various dyes, nor is any relation expressed between the region stained and the effect on ciliary movement.

Permeability to oxidation-reduction indicators and respiration

The various vital processes are so intimately related that it is difficult to isolate a set of experiments on the subject of permeability in which the penetration of dyes is studied, without considering their effects in other connections. For example, the oxidation-reduction dyes have been used by LUND (1928), LUND and KENYON (1928) and MARSH (1928) in a series of experiments up to the present date to show the relation between the penetration of these dyes, the oxidation-reduction potential and the respiration of the organism.

GENEVOIS (1928) was the first to recognize the relation between the oxidation-reduction potential of dyes, their penetration

and its effect upon O_2 consumption. He used a series of indicators beginning with thionine in the scale and ending with those of low reducing intensity, such as neutral red and safranin. He found that those with a low rH which penetrated the cells did not cause an increase in respiration, whereas methylene blue and thionine, which penetrated readily and have a higher potential, caused a considerable increase.

BARRON and HOFFMAN (1930), using the same method, also showed this relation between redox dyes and their effect on O_2 consumption in starfish eggs, extending the series of indicators to include the indophenols, and showing again that those that did not penetrate had little effect; that the maximum effect was obtained by those dyes having an rH around that of methylene blue, and that there was no effect by those dyes having a low rH.

Relation between Penetration of Oxidation-reduction Dyes and the Effects of KCN. A few experiments have been done in an endeavor to throw some light on the effects of KCN on the penetration and potential of the oxidation-reduction dyes. BARRON and HAMBURGER (1932) showed that KCN does not affect the catalytic action of dyes even in the absence of hemoglobin. CHAMBERS, BECK and GREEN (1933) showed that concentrations of KCN from N/10 to N/100 have no effect on the rate of reduction of these dyes by starfish eggs.

ROSS (1938) has made some direct observations on the relation between the concentration of methylene blue in the sap of *Nitella* at equilibrium in the presence and in the absence of cyanide, and the wet weight of the plant. These show practically no difference in the two cases. Table XXXVII gives these figures (Taken from Ross 1938).

MAKAROV (1934), on the other hand, in some experiments with frogs, in which he fed them methylene blue, janus green or neutral red and then noted the color of the cells in the intestine, found that with HCN there was no reduction of the dyes which were normally reduced, and that the ability to accumulate methylene blue by the intestinal cells was lost. This experiment is roughly qualitative and there seems to be no control for pH.

BROOKS (1938) found that when *Valonia* cells were placed in solutions of oxidation-reduction indicators containing KCN there was no change from the normal in either the rate of penetration or the power of reduction provided the pH was controlled. This

Table XXXVII. Amounts of methylene blue taken up

Original Conditions	Final Conc. of MB	Milli-equivalents taken up per gm. wet weight
10^{-4} M MB alone (4 hrs)	2.0×10^{-5} M	0.43
10^{-4} M MB + 10^{-3} M NaCN (4 hrs) .	3.2×10^{-5}	0.35
10^{-3} M NaCN + 10^{-4} M MB (2 hrs) .	2.4×10^{-5}	0.40
10^{-3} M NaCN + 10^{-4} M MB (2 hrs) .	2.4×10^{-5}	0.40
10^{-3} M NaCN & 10^{-4} M MB (2 hrs) .	2.6×10^{-5}	0.39
10^{-3} M MB alone (4 hrs)	7.9×10^{-5}	5.0
10^{-3} M MB + 10^{-3} M NaCN (4 hrs) .	5.8×10^{-5}	5.1
10^{-3} M NaCN + 10^{-3} M MB (2 hrs) .	5.7×10^{-5}	5.1
10^{-3} M NaCN + 10^{-3} M MB (2 hrs) .	6.0×10^{-5}	5.0

was true for one of the indicators from each region of CLARK's scale, namely, the very positive, the neutral and the negative at the same pH values. Only the dyes that have a positive or neutral electrode potential in the region of biological pH values have been found to be efficient antidotes for the effects of KCN in other cells. Since KCN had no effect on the rate of penetration of these dyes in the case of *Valonia*, it would appear that there is (1) either no direct relation between the permeability of the protoplasm of plant cells to these dyes and the oxidation-reduction mechanism, (2) that all the dyes were able to function as antidotes of KCN, or (3) that KCN did not affect these plants.

On the other hand, in the case of some experiments with animal cells, the relation between oxidation-reduction and "secretion" by the kidneys is shown by the use of various poisons which act upon specific steps in the process. BECK, KEMPTON and RICHARDS (1938) used cyanide in perfused frog kidneys and found that the arteries of the kidney become constricted; this produces a decrease in volume of blood in the glomeruli and an increase in their permeability.

Another case is that of the inhibition by cyanide and carbon monoxide of phenol red accumulation in the lumen of the segments of the proximal tubules of the kidneys as found by CHAMBERS (1935). These poisons act upon the respiratory enzyme which carries oxygen. It would have been interesting to know whether the addition of methylene blue, which is an antidote for the action

of cyanide and carbon monoxide, would permit the absorption of these dyes.

Another link in the oxidative chain is affected by monoiodoacetic acid which stops lactic acid formation. When this substance is used in experiments on intestinal absorption by glucose, inhibition of glucose absorption takes place, according to VERZÁR and WIRZ (1937) and WILBRANDT and LASZT (1933).

In conclusion, although it is frequently stated that methylene blue does not stain living protoplasm, with the implication that it does not penetrate, it should be noted that absence of color in the case of an oxidation-reduction dye should not be the criterion of penetration. Many of these dyes are colorless in the reduced form. The experiments of M. M. BROOKS (1932) with methylene blue show that the presence of the dye vitally affected the protoplasm. In these experiments methylene blue was used in the presence of cyanide and carbon monoxide, first with *Valonia* and then with rats, and its antidotal action noted with suggestions that methylene blue be used in clinical medicine in the case of these two poisons. These experiments, therefore, show that the dye acting as a catalyst for accepting hydrogen, enabled the cells to carry on respiration, while the dye itself was alternately oxidized and reduced. Therefore, in this case the dye became incorporated in the protoplasm by forming a link in the chain of oxidations.

Significance of oxidation-reduction potentials (rH)

In making a general survey of the different results obtained by various investigators using different methods, it is found that the rH under aerobic conditions varies from 12 to 22. These figures are the maximum range which could be obtained, assuming that the pH of protoplasm would be designated between 5 and 8 and the E_h between 0.1 and 0.2 volt. In the same way the range obtained by various investigators for oxidation-reduction potentials under anaerobic conditions is from 0 to 10 or 12. One might conclude that refinements of technique and elimination of those methods which give obviously misleading results would reduce the spread of 10 points in each case to a more uniform value.

No attempt has been made in this chapter to discuss mechanisms of oxidation-reduction nor the large literature which has been built up around this subject.

It has been shown that the range of rH is considerable in the experiments reported; that aerobic conditions produce a

higher rH than anaerobic conditions; that the electrode potential determines whether or not a substance is reduced or oxidized in the organism; that the redox potential of a solution determines growth of organisms suspended therein; and that the oxidation-reduction potential of an indicator is not the factor determining penetration into living cells.

All data which can be obtained on identifying the substances in the cell which may influence penetration into the cell, or which may throw some light on the state of the cell are important in the study of the laws regulating permeability.

According to GREEN, STRICKLAND and TARR (1934), if a series of indicators is microinjected into cells or added to cell suspensions, it is found that there is a potential level below which indicators are not reduced (NEEDHAM and NEEDHAM 1925). Aerobically this level is fairly positive in potential, anaerobically fairly negative (in starfish eggs, for example, the values are -0.06 and -0.17 volt, CHAMBERS et al. 1933); the two levels are called the aerobic and anaerobic potential respectively.

WURMSER (1932) considers oxidation-reduction potentials as a measure of the free energy of biological reactions; whereas MACHLIS and GREEN (1933) and BECK and ROBIN (1934) believe that the so-called "aerobic potential" and the "anaerobic potential" of the cells are determined purely by kinetics and have no thermodynamic significance. Suppose that methyl viologen is added to liver cells saturated with the substrates of the various dehydrogenase systems. Xanthine oxidase, formic dehydrogenase, etc., will reduce the dye to some extent, but more positive enzyme systems such as the lactic and succinic dehydrogenases will reoxidize the reduced methyl viologen. The above authors believe that the indicator will remain in the reduced or oxidized form as determined by the relative speeds of oxidation and reduction, and will not be directly connected with the potential.

It has also been found that two different indicators introduced into the same system sometimes indicate different potentials. For example, in one experiment with the system hypoxanthine-fumarate Nile blue was about 95% reduced, indicating a potential of about -0.20 volt, while benzyl viologen was 50% reduced, indicating a potential of about -0.36 volt. The authors believe that the ratio of rate of oxidation by fumarate over rate of reduction by hypoxanthine was not so much greater with benzyl viologen than with Nile blue as indicated by the experiments

In these experiments various factors were found to influence the rate of oxidation-reduction; namely, the specificity of the dye acting as a catalyst, the activity of the enzyme, the potential of the dye introduced and its concentration.

It seems to the writers that if the catalytic nature of the dye is important in giving indications of potentials, then obviously, since these are specific, values obtained cannot be regarded as indicating electromotively active substances. And in these cases the use of these dyes must be restricted.

The dilemma in this case seems to be that one dye oxidizes the system faster than the other reduces it and this influences the color of the dyes, so that the dye does not indicate the true equilibrium condition. If electrometric measurements by means of electrodes could be made in such cases, these would afford checks on experiments with dyes, and one could find out what the true values would be. The use of overlapping indicators should obviate these differences so that true potentials could be obtained with indicators, provided the indicator does not react chemically with the system used. COHEN (1933) states that when suitable indicators were injected into plant and animal cells under aerobic and anaerobic conditions, the rH was approximately the same for the same indicators in the different cells.

In another paper MACHLIS and GREEN (1934) state that a system containing cytochrome, a dehydrogenase system and an oxidase would assume a potential which has no real significance, and that the potentials so measured do not correspond to an intrinsic property of the cell. It seems to the writers that such a combination of systems is no different from that of a similar analogy with acid-base indicators. If one adds different acids and bases together, the resultant will be a value which has real significance. In the same way, if a number of oxidation-reduction systems are added together, there will be a resultant of all these values which should also have real significance. The use of electrometric methods as checks on potentials obtained with dyes should be the answer as to whether or not indicators can be used to obtain oxidation-reduction potentials in doubtful cases. If such checks are made then we can keep CHAMBERS (1934) definition of the "values so obtained as an average between relative activities of the reducing and oxidizing reactions within the cell".

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p. 268, For KOOHES read KOVACS.

p. 334, insert: HEVESY, G. VON, and HOFER, E. (1934). Der Austausch des Wassers im Fischkörper. Hoppe-Seylers Z. physiol. Chem. 225: 28—34.

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